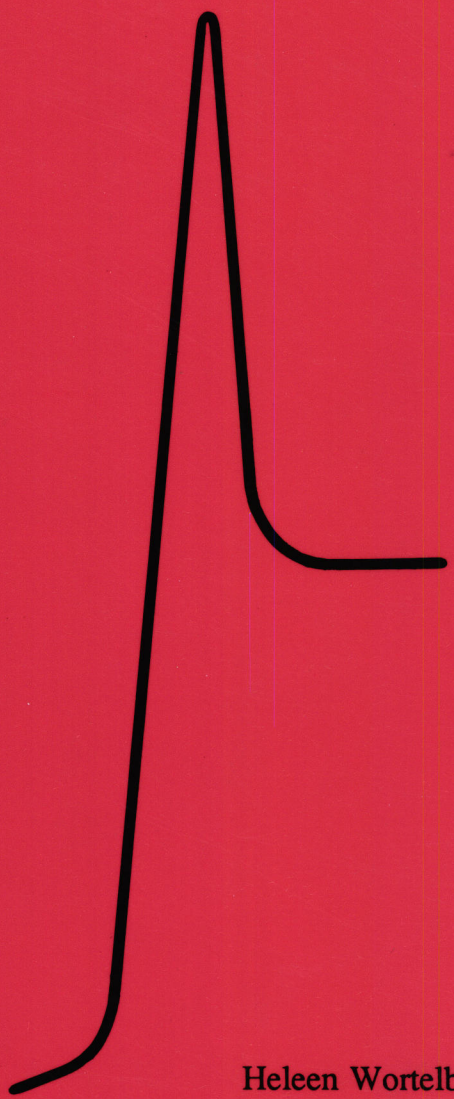
PRIMARY HEPATOCYTE CULTURES AS A MODEL SYSTEM FOR THE DETERMINATION OF INDUCTION OF BIOTRANSFORMATION ENZYMES

Effects of glucosinolate hydrolysis products



Heleen Wortelboer

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PRIMAIRE HEPATOCYTEN CULTURES ALS EEN IN VITRO MODEL TER BEPALING VAN INDUCTIE VAN BIOTRANSFORMATIE ENZYMEN

Effecten van hydrolyse produkten van glucosinolaten
(met een samenvatting in het Nederlands)

PROEFSCHRIFT

Ter verkrijging van de graad van doctor aan de
Rijksuniversiteit te Utrecht, op gezag van de
Rector Magnificus Prof. Dr. J.A. Van Ginkel,
ingevolge het besluit van het college van
dekanen in het openbaar te verdedigen op
woensdag 2 oktober 1991 des namiddags te 2.30 uur

door

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geboren 17 mei 1958, te Den Helder

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Het in dit proefschrift beschreven onderzoek werd gefinancieerd door het UTOX, een samenwerkingsverband tussen het Research Instituut voor Toxicologie (RITOX) te Utrecht, TNO-Voeding (TNO-ITV) te Zeist en het Rijksinstituut voor Volksgezondheid en Milieuhygiëne (RIVM) te Bilthoven.

Contents

Abbreviations

AH	aniline 4-hydroxylation
BII	2,3-bis [3-indolylmethyl] indole
BNF	β-naphthoflavone
CLOF	clofibrate
CLOFA	clofibric acid
COSY	correlation spectroscopy
CTI	5,6,11,12,17,18-hexahydro-cyclonona [1,2-*b*:4,5-*b'*:7,8-*b''*] tri-indole
DEX	dexamethasone
DIM	3,3'-diindolylmethane
DMSO	dimethyl sulfoxide
DTD	DT-diaphorase (= NAD(P)H-quinone oxidoreductase)
EDTA	(ethylenedinitrilo)tetraacetic acid
EROD	7-ethoxyresorufin O-deethylation (= 7-ethoxyphenoxazone O-deethylation)
GST	glutathione S-transferase
GT	UDP-glucuronyl transferase
HBSS	Hanks' balanced salt solution
HPLC	high performance liquid chromatography
I3A	indole-3-acetonitrile
I3C	indole-3-carbinol
I3CA	indole-3-carboxaldehyde
INH	isoniazid
i.p.	intraperitoneally
LDH	lactate dehydrogenase
1-NG	1-naphthol glucuronide
NMR	nuclear magnetic resonance
1-NS	1-naphthol sulphate
MS	massa spectroscopy
OHT	hydroxytestosterone
P450	cytochrome P450
PB	phenobarbital
PBS	phosphate-buffered saline
p.o.	per os
PROD	7-pentoxyresorufin O-dependentylation (= 7-pentoxyphenoxazone O-dependentylation)
PVDF	polyvinylidene difluoride
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gelelectrophoresis
ST	sulfotransferase
TBST	Tris-buffered saline containing 0.3% Tween 20
TCA	trichloroacetic acid

CHAPTER 1

GENERAL INTRODUCTION

1. Biotransformation

Through the intake of diet, drugs and air, organisms are exposed to a vast number of compounds such as natural food compounds, pesticides, industrial chemicals, pollutants etc. Not all of these environmental chemicals (xenobiotics) are beneficial, and some have marked deleterious effects. The duration of action of a biologically active compound is dependent on absorption, on tissue distribution and reaction with specific cellular receptors, and on the rates of metabolic deactivation and elimination from the body. The metabolism of xenobiotics (biotransformation) is regulated by several enzyme systems (Jakoby, 1980). The liver is quantitatively the most important organ in this respect, but many other tissues, such as the small intestine (Hoensch and Schwenk, 1984; Noordhoek, 1987), kidney (Anders, 1980), lung (Kramer *et al*, 1985) and skin (Noonan and Wester, 1983) also contribute to the overall metabolism of chemicals.

Biotransformation of xenobiotics may be considered to occur in two phases. Phase I metabolism involves reactions like oxygenation, oxidation, reduction, dehalogenation, hydrolysis which introduce new functional groups into the lipophilic molecules, thereby making them more hydrophilic and more readily excretable. The most important phase I enzymes are the cytochrome P450 enzymes. Phase II metabolism (conjugation enzymes) comprises synthetic reactions in which small endogenous molecules (glucuronic acid, glutathione, sulphate, glycine and other amino acids) are added to the functional groups of the xenobiotic or its phase I metabolite, making them even more polar and readily excretable. A compound may be metabolized by either one or both phases of metabolism (Jakoby, 1980).

Biotransformation should not only be considered as a detoxication process. Many examples are known in which a biologically inert compound is transformed into a potentially toxic one. The formation of a more toxic metabolite from a less toxic parent compound is called bioactivation. Examples are the bioactivation of procarcinogens, such as benzo[*a*]pyrene (Gelboin, 1980), aflatoxin B_1 (Ishii *et al*, 1986), 7,12-dimethyl-benz[*a*]anthracene (Vigny *et al*, 1985) by specific cytochrome P450s, but also the bioactivation of 1,2-dibromoethane by glutathione S-transferase, and N-hydroxy-2-acetylaminofluorene by sulphation (van Bladeren *et al*, 1987). In succession, the toxic metabolites can be detoxified by other biotransformation enzymes. Ultimately, it is the balance between bioactivation and detoxication which primarily determines whether a reactive metabolite can elicit its toxic effect or not.

Biotransformation enzyme activities can be elevated and depressed by various xenobiotics, among them food compounds, pesticides, and drugs, thereby affecting the metabolism and toxicity of its own or other xenobiotic agents (Conney, 1986). Over the last two decennia it became clear that several forms of cytochrome P450 exist, each exhibiting different catalytic activities and being inducible by different chemicals. Changes in this cytochrome P450 pattern play an important role in the balance between activation and detoxication of potentially toxic xenobiotics. To date, only limited knowledge has been gathered on which - and to what extent - compounds are capable in changing these cytochrome P450 profile.

2. Cytochromes P450

Among the phase I enzymes the most widely studied is the cytochrome P450 system, probably due to the tremendous variety of substrates that can be metabolized (for review see Guengerich, 1990). Apart from cytochrome P450s, other phase I enzymes such as alcohol and aldehyde dehydrogenases (Jakoby, 1980), flavin-containing monooxygenase (Ziegler, 1988) and prostaglandin synthase (Eling *et al*, 1990) are capable of (co)oxidation of xenobiotics, too. Prostaglandin synthase has generally high activity in organs with low cytochrome P450 activity. It probably plays a significant role in the metabolic activation of polycyclic aromatic hydrocarbons in tissues such as lung and colon. Furthermore, prostaglandin synthase has been suggested to be involved in the bioactivation of certain kidney and bladder carcinogens, such as the aromatic amines benzidine and naphthylamine.

Cytochrome P450 is a monooxygenase (mixed-function oxidase) which incorporates one atom of molecular oxygen into an organic substrate while using reducing equivalents (NADPH) to reduce the other atom of molecular oxygen to water. The monooxygenase system is localized in smooth endoplasmic reticulum and mitochondria and are embedded in the membranes of these organelles. The system is composed of the haemoprotein cytochrome P450 together with an electron donating system consisting of NADPH-P450 reductase, cytochrome b_5 reductase and cytochrome b_5. The phospholipid environment in the membrane is important for the optimal interaction between the different proteins (Ingelman-Sundberg, 1986). The cytochrome P450 system can also produce H_2O_2 and superoxide anions which may cause cellular damage. Next, P450s are capable of catalyzing oxygenation reactions using the oxygen atom of a peroxide substrate (Ortiz de Montellano, 1986). Certain substrates, e.g. N-oxides and azo compounds can be reduced by NADPH-P450 reductase or P450 to the corresponding amines and hydrazines (Wislocki *et al*, 1980). Usually a low oxygen tension favours the reductive pathways (Ahr *et al*, 1982; MacDonald, 1982).

2.1. Multiplicity and nomenclature of P450 enzymes

Thirty five years ago the first reports in which cytochrome P450 was involved (although unknown at that time) were published. Several authors reported the inhibition of carcinogenicity of an aminoazo dye due to an enhanced metabolism of this compound

in rats treated with 3-methylcholanthrene (Conney *et al*, 1956). Klingenberg (1958) and Garfinkel (1958) were the first to identify a pigment which in reduced form exhibits an intense absorption at 450 nm after binding to carbon monoxide. The pigment was further characterized as a haemoprotein by Omura & Sato (1962, 1964) and they called it cytochrome P450 (pigment absorbing at 450 nm).

Over the next 20 years it became apparent that cytochrome P450 plays a crucial role in the metabolism of many endogenous and xenobiotic compounds (Conney, 1986), and that not one, but multiple forms of this monooxygenase are involved. At first, two major forms were characterized by its specific maximum absorption in the CO-binding assay, i.e. cytochrome P450 (inducible by phenobarbital) and P448 (inducible by 3-methylcholanthrene) (Alvares *et al*, 1967). Later Lu *et al* (1978) reported that cytochrome P450 fractions partially purified from livers from phenobarbital and 3-methylcholanthrene-treated rats markedly differed in catalytic activities compared to P450 fractions from untreated rats. Thereafter, many other forms of cytochrome P450 were purified, which resulted in the development of polyclonal and monoclonal antibodies to characterize the protein levels and specific enzyme activities. Subsequently, antibodies were used in the isolation and characterization of many P450 cDNAs and their genes.

The purification and sequencing of multiple P450 proteins in different laboratories (Cheng and Schenkman, 1982; Guengerich *et al*, 1982; Ryan *et al*, 1982; Ryan *et al*, 1984; Tamburini *et al*, 1984; Wrighton *et al*, 1985) resulted in numerous trivial names for the same P450 protein. In 1987 several authors proposed a standardized P450 gene nomenclature, based on the similarities between the amino acid coding sequences (Nebert *et al*, 1987). Much more different P450 forms were purified. While in 1987 a mere 31 different P450 genes were described, an updated list of 71 genes was presented only two years later (Nebert *et al*, 1989). In October 1990 the "P450 superfamily" consisted of 154 different genes, which have been described in a total of 23 eukaryotes (including nine mammalian and one plant species) and seven prokaryotes (Nebert *et al*, 1991; Fig 1).
Within the P450 superfamily genes are assigned to families and subfamilies. In general, within a single family, the P450 protein sequences are >40% identical. Within the same subfamily the mammalian P450 protein sequences are >55% identical, though the inclusion of genes of more distant species, e.g. trout 1A1 drops this value to >46%. An "orthologous gene" in two species refers to a gene of which isknown to correspond to an ancestral gene which existed before the evolutionary divergence of the two species. In several subfamilies (e.g. 2C) numerous species-specific changes in the genes have made orthologous assignment between species impossible; these "non-orthologous" genes within one subfamily are sequentially numbered. At the latest conference of Microsomal Drug Metabolism (Stockholm, 1990) R.W. Estabrook speculated that over 200 to 300 different genes will be identified. Parallel with the number of genes, an increasing list of reviews on this important enzyme system and its characteristics has been published.

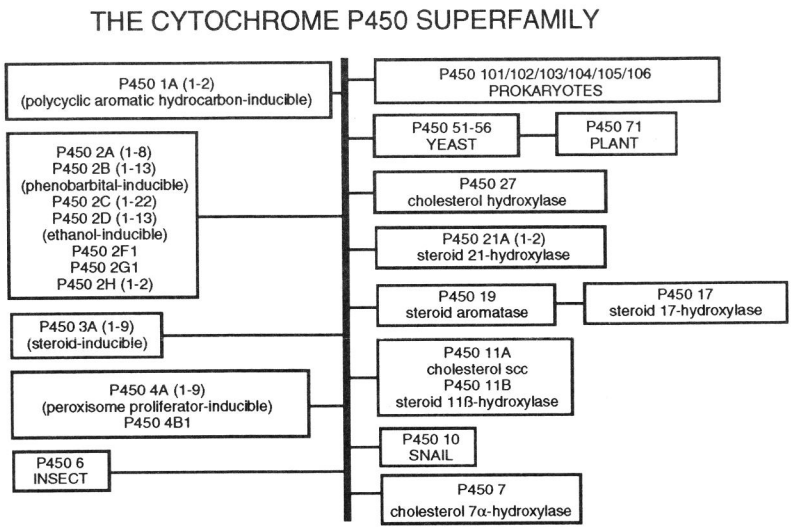

Fig. 1. The families of P450s as described by Nebert *et al* (1991). Assignment to a specific family is based on protein sequence similarity (within one gene family: >40% identical). The numbers in the brackets indicate the designated numbering for identified members of each family or subfamily. (Adapted from Estabrook *et al*, 1990).

2.2. Catalytic properties of mammalian cytochrome P450 enzymes

Of the 27 gene families so far described, 10 exist in all mammals. The mammalian P450s can be divided in P450 proteins mainly involved in xenobiotic metabolism (with often overlapping substrate specificity; families 1 to 3), and those involved in physiological metabolism i.e. the metabolism of fatty acids (family 4) and biosynthesis of sterols (exhibiting a high degree of regio- and stereospecificity; families 7, 11, 17, 19, 21, and 27).

P450s involved in physiological metabolism

The oldest cytochromes P450, evolutionary spoken, are the P450s involved in the metabolism of cholesterol (P450 7 and 11) and fatty-acids (P450 4), and those involved in the synthesis of steroids (P450 17, 19, 21, and 27). The biochemical regulation of steroidogenic cytochromes P450 is far more stringent because of the physiological importance of these steroid hormones. The different forms are distributed between mitochondria and endoplasmic reticulum and are located in a variety of tissues (Table 1). The mitochondrial P450s are distinct from the ones in the endoplasmic reticulum in that they collect electrons via adrenodoxin and adrenodoxin reductase (For reviews see Fevold, 1983; Jefcoate, 1986).

Table 1: Distribution of steroidogenic cytochromes P450 between mitochondria and endoplasmic reticulum.

P450	Reaction[a]	Substrate	Tissues
Mitochondria			
11A	20,22-cleavage	cholesterol	adrenal, testis, ovary, placenta
11B	11-β, 18-OH	deoxycorticosterone	adrenal
	18-OH	corticosterone	adrenal glomerulosa
27	1α-OH	25-hydroxyvitamin D3	kidney
	6-OH	cholesterol	liver
Endoplasmic reticulum			
21	21-OH	(17α-hydroxy)progesterone	adrenal
17	17α-OH	progesterone	adrenal, testis
19	aromatase	androgens	ovary, placenta
7	7α-OH	cholesterol	liver
?	25-OH	vitamin D	liver
?	12α-OH	dihydroxycholestane	liver
?	25-OH	trihydroxycholestane	liver
?	14α-OH	dihydroanosterol	liver

[a] OH, hydroxylation
(Adapted from Jefcoate, 1986).

Xenobiotic-inducible P450s

A clear physiological function has not been demonstrated for cytochrome P450s in families P450 1 through 4, except for the fatty acid *w*-hydroxylation by P450 4 (Gibson *et al*, 1982). In contrast, these P450 families are involved in the metabolism of various chemically different compounds and their catalytic activities can be markedly enhanced. Therefore, these P450s are of great interest in pharmacology and toxicology studies.

The multiple P450 forms are most thoroughly studied in the rat. Studies using systems of purified P450 proteins reconstituted with phospholipids and NADPH-cytochrome P450 reductase (Guengerich *et al*, 1982) revealed that individual forms of P450 can exhibit "highly specific" and "less specific" catalytic activities towards several different substrates. For example, a single P450 form can possess a high K_m activity toward one or more substrates and a low K_m activity toward other substrates. Furthermore, a single substrate such as testosterone can be metabolized by several forms of P450 at different sites (Table 2).

Table 2: Substrate selectivities of purified rat hepatic cytochromes P450

Substrate	Reaction[a]	Cytochrome P450 enzyme											
		1A1	1A2	2A1	2B1	2B2	2C6	2C7	2C11	2C12	2C13	2E1	3A1
		nmol/min/nmol P450											
benzphetamine	*N*-demethyl.	2.3	132.5	6.7	3.9	19.8	14.1	1.3	52.1	2.8	4.9	5.5	*
benzo[*a*]pyrene	3/9-OH	-	-	23.4	-	-	-	-	1.8	-	-	-	*
7-ethoxycoumarin	*O*-demethyl.	0.6	9.6	97.1	0.6	2.0	0.5	-	0.9	0.7	1.1	1.2	*
7-ethoxyresorufin	*O*-deethyl.	6.9	0.5	-	-	-	-	*	-	*	*	*	-
aniline	4-OH	-	1.8	1.0	9.6	-	1.0	-	1.5	-	-	12.7	*
nitrosodimethyl-amine	*N*-demethyl.	-	-	-	0.5	-	0.5	-	1.1	-	-	*	15.9
testosterone	7α-OH	20.9	-	-	-	-	-	-	-	-	-	-	-
	16α-OH	-	9.1	-	-	0.8	-	0.8	7.9	-	-	-	-
	16β-OH	-	7.2	-	-	0.7	-	-	-	-	-	-	-
	6β-OH	-	-	1.9	0.7	-	-	-	-	-	3.8	-	3.1
	2α-OH	-	-	-	-	-	-	-	7.3	-	-	-	-

Adapted from Guengerich *et al* (1982), Conney (1986), Sonderfan *et al* (1987) and Levin (1990) and refs therein. [a] OH, hydroxylation. (-) activity < 0.5 nmol/min/nmol P450; (*) activity not determined.

Obviously, these studies using purified enzymes are informative with respect to the catalytic competence of a given form for a particular substrate, but they do not provide definitive information on the contribution of one or more enzymes to the metabolism of a substrate by intact microsomes, and certainly not in intact cells. Differences in K_m, lipid requirements, competition for reductase, membrane orientation, and contribution of other factors such as cytochrome b_5 can all be factors determining the ultimate activity. With the use of (inhibitory) monospecific antibodies several catalytic activities could bemore specifically subscribed to one or two P450 enzymes in microsomal preparations (Levin *et al*, 1987; Waxman, 1988). In this respect, excellent studies of Nakajima *et al* (1990) revealed that although a catalytic activity might be subscribed to one P450 form after induction of this form, e.g. 7-ethoxyresorufin O-deethylation by P450 1A1, in untreated rats, with only low expression of P450 1A1, this activity is catalysed by the constitutive P450 2C11 and 2C6.

2.3. Human P450s

The chemistry, regulation and biology of cytochromes P450 are most extensively studied in rat and mice. Human P450s have been studied only during the last ten years and they have been purified from human liver by several laboratories (Wang *et al*, 1983; Guengerich *et al*, 1986; Miles *et al*, 1988). Among the P450 superfamily rat and human orthologous genes have been found predominantly in the steroidogenic P450 families, i.e. 7, 21A, 21B, 17, 19, and 27 (Nebert *et al*, 1991). Throughout P450 families 2 to 4, however, the several genes are much more diverse. A list of rat and human P450s among P450 families 1 to 4, and their known characteristics substrates are given in Table 3.

Table 3: Rat and human P450 forms in the families 1, 2, 3 and 4.

P450 subfamily	rat	human	characteristic substrates	characteristics
1A	1	1	7-ethoxyresorufin	human 1A1 mainly extrahepatic
	2	2	phenacetin; 7-methoxyresorufin	
2A	1,2,3		testosterone (7α)	
		6/7	?	
2B	1,2,3		7-pentoxyresorufin; testosterone (16β)	
		6	?	
2C	6,7			
		8,9,10	S-mephenytoin	
	11		testosterone (2α; 16α)	rat male specific
	12		testosterone (15β)	rat female specific
	13			rat male specific
		17,18,19	?	
	22,23		?	
2D	1,2,3,4,5		debrisoquine	
		6	debrisoquine	
2E	1	1	aniline, ethanol	
2F		1	?	human lung tissue
2G	1		?	rat olfactory tissue
3A	1,2		testosterone (6β; 15β)	
		3,4,5	nifedipine, testosterone (6β); cortisol (6β)	
		7	?	human foetal liver
	9		?	rat olfactory tissue
4A	1,2,3		lauric acid	
		9	lauric acid	
4B		1	?	human lung tissue

(Obtained from Nebert and Gonzalez (1987) and Nebert *et al* (1991)).

A major problem studying human P450s is the large interindividual variability, due to either genetic differences or differences in diet and xenobiotic exposure. The enzymes P450 1A2, 3A (representing one or more proteins) and 2E1 are easily detectable in human liver (Wrighton *et al*, 1986; Wrighton *et al.* 1987; Waxman *et al*, 1988), but the immunochemically detectable levels can vary up to 10-fold. P450 1A1 is undetectable in most human livers (Wrighton *et al*, 1986), but the enzyme has been detected in the placenta of smokers (Sesardic *et al*, 1990).

Profound genetic differences exist in the expression of P450 2D and the associated 4-hydroxylation of debrisoquine. About 5% of the Caucasian population exhibits the "poor metabolizer" phenotype. At least 20 other drugs are now known to be metabolized by P450 2D (Wolff *et al*, 1985). Another human genetic polymorphism is found in the

expression of P450 2C proteins, associated with the hydroxylation of the anti-epileptic drug mephenytoin. The "poor-metabolizing" phenotype occurs with lowfrequency in Caucasians (*ca* 3%), but with considerably higher frequency (> 20%) in the Japanese population (Guengerich, 1989).

Only limited data are available concerning the regulation of human P450s. Cultures of human cells are increasingly providing adequate means to study the regulation of human cytochrome P450s (Cresteil *et al*, 1987; Donato *et al*, 1990; Morel *et al*, 1990).

3. Phase II enzymes

Many reactive intermediates formed via the cytochrome P450 system (and other phase I enzymes) can be further metabolized by phase II enzymes through conjugation with endogenous ligands, such as glucuronic acid, glutathione and sulphate. Similar to cytochromes P450, conjugating enzymes consist of enzyme families with distinct but overlapping substrate specificity.

UDP-glucuronyl transferases (GT, EC 2.4.1.17) form a multigene family of proteins that catalyses the transfer of UDP-glucuronic acid to a suitable acceptor molecule, forming the glucuronide conjugate (Burchell *et al*, 1987; Bock *et al*, 1987). Nine different GTs have been identified, of which six have been purified. Three of the transferases are more specific for the conjugation of endogenous substrates such as bilirubin (bilirubin-GT), steroid hormones (3α- and 17β-hydroxy steroid GTs) and estradiol (aromatic steroid-GT). Others are involved in the glucuronidation of several xenobiotics i.e. 4-nitrophenol-GT (GT1; 3-methylcholanthrene-inducible), GT2 (phenobarbital-inducible) and foetal phenol-GT. Glucuronide formation is quantitatively the most important form of conjugations of xenobiotics and endogenous compounds. The GT enzymes are mainly localized in the membrane of the smooth endoplasmic reticulum and are therefore ideally positioned to glucuronide the products of mixed function oxidase reactions. Hepatic glucuronide conjugates are often excreted in bile and thus released into the intestinal tract where they can be broken down to the parent compound by β-glucuronidase and possibly reabsorbed (enterohepatic circulation).

Glutathione S-transferases (GST, EC 2.5.1.18) exist in multiple forms that conjugate glutathione on the sulphur atom of cysteine to various electrophiles (Mannervik, 1985; Pickett and Lu, 1989; Coles and Ketterer, 1990). In addition, GSTs bind a variety of hydrophobic compounds such as haem, bilirubin and polycyclic aromatic hydrocarbons with high affinity and have been suggested to act as intracellular transport proteins (Mannervik, 1987). Moreover, GSTs have been shown to play a role in the transport of hormones to the cell nucleus. The GSTs are cytosolic proteins, with the exception of a microsomal form that has been identified in rodents. The enzymes are homodimers or heterodimers comprising at least seven subunits. Until now, evidence exist for the occurrence of more than 11 subunits in the rat and 7 subunits in human (Coles and Ketterer, 1990). The GSTs are classified into three different families, named alpha, mu, and pi, respectively (Table 4).

GSTs are widespread in nature and have been found in almost every rat and human tissue examined. The expression of GSTs in different tissues, however, is not uniform. For example, in rat liver subunits 1, 2, 3 and 4 are predominant, whereas in the lung, small intestine and placenta subunit 7 is the major form. Furthermore, it has been suggested that expression of subunit 7 is a common phenotypic expression of malignant transformation of cells, including malignant liver cells.

Table 4: Rat and human glutathione S-transferases

Glutathione S-transferases		
Class alpha	Class mu	Class pi
rat 1-1	rat 3-3	rat 7-7
1-2	3-4	human π
2-2	4-4	
8-8	6-6	
human α	human μ	

(From Mannervik *et al*, 1987).

To determine the catalytic activity of GSTs the most commonly used substrate is 1-chloro-2,4-dinitrobenzene (CDNB), since it is utilized well by most of the rat and human subunits. Although the products of GST-catalysed reactions are usually non-toxic, the formation of glutathione conjugate with certain compounds may cause cell injury. For instance, the glutathione conjugate with 1,2-dibromoethane can react directly with DNA forming an adduct that may be mutagenic. Another type of enhanced toxicity is exerted via metabolism of glutathione conjugates of certain halogenated hydrocarbons within the kidney to cysteine conjugates. The cysteine conjugates may be further metabolized by a renal enzyme (β-lyase) to a sulphur-containing reactive compound which can initiate injury in proximal renal tubular cells (Coles and Ketterer, 1990).

Sulfotransferases (ST; EC 2.8.2.1) are capable of the formation of sulphate monoesters by reaction of 3'-phosphoadenosine 5'-phosphosulphate (PAPS) with compounds bearing the hydroxyl group. The enzymes are also involved in the biosynthesis of steroids and heparin (Jakoby *et al*, 1980). Sulfotransferases are located in the cytosol. Most compounds that can be glucuronidated can also be sulfated and this leads to the possibility of competition for the substrate between the two pathways. In general sulphate conjugation predominates at low substrate conjugation due to the kinetics of the two reactions and the limited availability of inorganic sulphate, and hence of PAPS.

Epoxide hydrolases (EH; EC 3.3.2.3) are monomeric proteins important in the degradation of potentially reactive epoxides by the conjugation of water (Oesch, 1980; Timms *et al*, 1987). Three different forms have been identified in the endoplasmic reticulum and one in the cytosol. The detoxication of the reactive and highly toxic epoxides to dihydrodiols results, in many instances, in a detoxication, but can, in other instances, serve as a precursor of even more reactive dihydrodiol epoxides. For example, carcinogenic diol-epoxides are formed of benzo[*a*]pyrene after a cytochrome P450-dependent formation to benzo[*a*]pyrene 7,8-oxide, a subsequent hydrolysis of the epoxide to (-)-7,8-dihydrodiol and finally a second P450-dependent oxidation to (+)-anti-7,8-diol-9,10-epoxide (Gelboin, 1980). In addition to metabolizing epoxide metabolites of xenobiotics, epoxide hydrolases catalyse the hydration of endogenous epoxides, such as oestrogen and androgen epoxide, and cholesterol epoxides (Timms *et al*, 1987).

DT-diaphorase or NAD(P)H:(quinone-acceptor)oxidoreductase (DTD, EC 1.6.99.2) is another widely distributed enzyme that protects cells against the toxicity of quinones and their metabolic precursors (e.g. polycyclic aromatic hydrocarbons, benzene) (Ernster, 1967; Smart and Zannoni, 1984). As such, DTD is not a conjugation enzyme, but may be appropriately considered a phase II enzyme because it does not introduce new functional groups and is generally induced coordinately with other phase II enzymes (Prochaska *et al*, 1985).

4. Regulation of biotransformation enzymes

The expression of genes encoding enzymes can be regulated at several different levels e.g. transcription of DNA; processing, stabilization and transport of mRNA; translation of mRNA and enzyme function. The gene expression of biotransformation enzymes may be affected at different levels by several factors: For instance, intrinsic factors, such as sexe and age, which determine the expression of the "constitutive" forms of different biotransformation enzymes (Skett, 1988). Furthermore, selective expression of biotransformation enzymes is known to occur in the presence of disease: For example the selective expression of P450s in the case of diabetes and hypertension (Schenkman *et al*, 1989), and the selective expression of GST (class pi enzyme) in neoplastic tissues (Pickett and Lu, 1989). There are, on the other hand, exogenous factors which can also have a profound effect on biotransformation, especially those agents which induce drug-metabolizing enzymes.

4.1. Enzyme induction

"Induction" implies synthesis of new enzymatically active protein, but it is sometimes used more broadly (especially in older literature) to denote stimulation of enzyme activity. The induction of a specific P450 is associated with increased biosynthesis of the haemoprotein, which requires *de novo* synthesis of apoprotein, but not of haem, since the haem pool can usually meet the increased requirement (Padmanaban *et al*, 1989). There are, on the other hand, indications that haem can modulate the

transcription of P450 genes. For instance, inhibition of haem synthesis or stimulation of haem degradation blocks the induction of 2B mRNA by phenobarbital (Bhat and Padmanaban, 1988).

Induction of a P450 enzyme activity can be over 50-fold, whereas the induction of phase II enzymes is lower (up to *ca* 3 to 5-fold). Many compounds of widely diverse chemical structures are able to induce biotransformation enzymes. A list of inducers of various biotransformation enzymes is given in Table 5. Some of these compounds are widely used as "model inducers" for their specificity to induce one particular cytochrome P450 to very high levels. For example, treatment of rats withβ-naphthoflavone or 3-methylcholanthrene results in an almost selective induction of P450 1A1 to very high levels. On the other hand, exposure of rats to the sedative drug phenobarbital results in very high levels of the P450 2B1 form, whereas the hypolipidaemic drug clofibrate enhances specifically P450 4A. In addition, compounds we are regularly exposed to, such as the persistent environmental contaminants PCDDs and PCBs (Safe *et al*, 1985), and natural constituents in edible plants (indoles, dithiolthiones, thiocyanates, flavonoids) are potent inducers of biotransformation enzymes, too (Guengerich, 1984).

Table 5: Inducers of biotransformation activities in animals.

Class of compounds	Example	Use or occurrence
Drugs	phenobarbital and most barbiturates	sedative/hypnotic
	pregnenolone-16α-carbonitrile	catatoxic steroid
	rifampicin	antibiotic
	triacetyloleandomycin	antibiotic
	clofibrate	hypolipidaemic
Alcohols	ethanol	beverage, skin desinfectant
Flavones	β-naphthoflavone	synthetics, citrus fruits
Food additives	butylated hydroxyanisole (BHA)	food antioxidants
	butylated hydroxytoluene (BHT)	
	ethoxyquin	
Anutrients	isosafrole	oils of nutmeg and cinnamon
	indoles	cruciferous vegetables
Halogenated hydrocarbons	2,3,7,8-tetrachlorodibenzo-*p*-dioxin	combustion product
	3,3',4,4'-tetrachlorobiphenyl	insulator capacitors/transformers
	3,3',4,4',5,5'-hexabromobiphenyl	flame retardant
Insecticides	DDT	agricultural pesticide
Polycyclic aromatic hydrocarbons	3-methylcholanthrene, benzo[*a*]pyrene	combustion products, cigarette
	chrysene, benzo[*a*]anthracene	smoke, oil contaminants
Solvents	toluene and xylenes	solvents, cleaning agents and degreasers

(Adapted from Gibson and Skett, 1986).

In general, chemicals are not particularly selective in inducing cytochromes P450 or phase II enzymes. For example, phenobarbital can induce next to P450 2B1, at least three other cytochromes P450 (2B2, 2A and 3A) as well epoxide hydrolase, glutathione S-transferase, UDP-glucuronyl transferase, and other enzymes (Waxman and Walsh, 1982; Wolf *et al*, 1984; Burchell *et al*, 1987). Clofibrate is known to induce at least two cytochromes P450, UDP-glucuronyltransferase and epoxide hydrolase (Tamburini *et al*, 1984; Burchell *et al*, 1987; Timms *et al*, 1987).

4.2. Mechanism of induction

The phenomenon of induction of biotransformation enzymes has been known for several decades. Only in the past few years, however, the mechanisms which regulate the induction process have been beginning to understand at the molecular level. Induction has been found to be due to both transcriptional and/or posttranscriptional regulation (Whitlock, 1986; Nebert and Gonzalez, 1987; Gonzalez, 1989).

4.2.1. Transcriptional mechanisms of induction

3-Methylcholanthrene-inducible P450s: the Ah receptor

From all P450s the mechanism of induction of P450 1A1 is the best understood, mainly due to four fortuitous circumstances a) the polymorphism for 3-methylcholanthrene induction in mice, b) a responsive cell system, c) the potent inducing agent TCDD and d) the ability to select cell-lines defective in induction of benzo[*a*]pyrene hydroxylase activity. As a result it has been shown that the P450 1A1 inducer, such as TCDD, 3-methylcholanthrene or β-naphthoflavone binds with high affinity with an intracellular cytosolic receptor protein designated the aryl hydrocarbon (*Ah*) receptor. The inducer-receptor complex is then translocated to nuclear chromatin, leading to transcriptional activation of a number of genes, such as P450 1A1 and P450 1A2, glucuronyl transferase (GT1; Owens, 1977), DT-diaphorase (Kumaki *et al*, 1977) and glutathione S-transferase 1-1 (Pickett and Lu, 1989). The induction of this *Ah* receptor programme strikingly resembles the mechanism of action of steroid hormones (Evans, 1988). Utilizing recent techniques of molecular biology, xenobiotic regulatory elements (XREs) and drug regulatory elements (DREs) could be identified upstream from the promotor (P) region of the structural gene of P450 1A1 (Pickett and Lu, 1989; Bock *et al*, 1990; Fig. 2). According to the high degree of nucleotide similarity in three different species (rat, mouse and man), it has been suggested that XREs and DREs cluster in two regions, at -500 kb and at -1000 kb upstream from the structural gene. The regulatory elements at -500 kb may be involved in negative control of gene expression after binding of a *trans*-acting repressor gene product (X). The regulatory elements at -1000 kb appear to be involved in MC-type induction. It has been suggested that also several phase II enzymes can be co-induced with cytochrome P450 1A1. The induction of glutathione S-transferase 1-1 requires the presence of functional *Ah* receptors (Pickett and Lu, 1989). It is unknown whether the inducer-*Ah* receptor complex interacts directly with responsive elements or indirectly through another *trans*-acting regulatory gene product (Y; Fig 2). A similar mechanism as described for the co-induction of P450 and GST has been postulated for the co-induction of P450 1A1 with glucuronyl transferase (GT1), DT-diaphorase and P450 1A2 (Gonzalez *et al*, 1984).

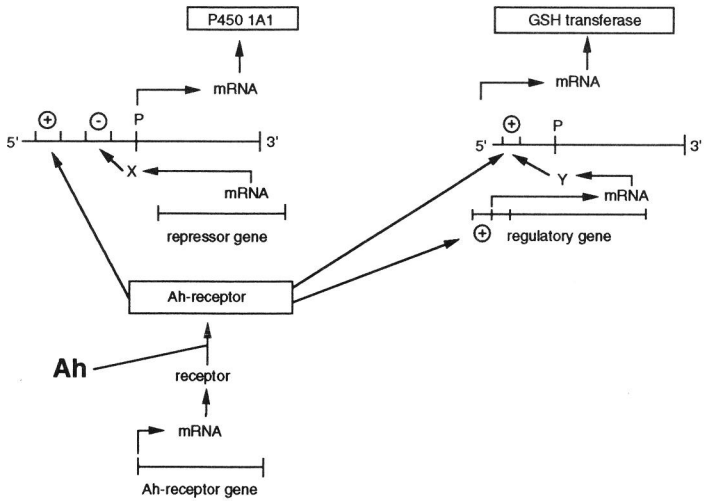

Fig. 2: Hypothetical scheme for co-induction by polycyclic aromatics of P450 1A1 and of conjugating enzymes such as glutathione S-transferase subunit 1.

The *Ah* receptor binds planar aromatic hydrocarbons (*Ah*) with high affinity. This ligand-*Ah* receptor complex is translocated to nuclear chromatin where it interacts with positive regulatory elements (+) upstream from the promotor (P) of the structural genes for cytochrome P450 1A1 and GST subunit 1. In addition to positive regulatory elements a negative regulatory element (-) has also been recognized. It is still uncertain whether the ligand-*Ah* receptor complex interacts directly with regulatory elements (+) of GST subunit 1 or indirectly through a still hypothetical protein (Y) (Adapted from Pickett and Lu, 1989)

To date, every mammalian species examined contains the *Ah* receptor, although sometimes with markedly different affinities for their ligands (Nebert and Gonzalez, 1987; Whitlock, 1987). This receptor difference, which is indicated as *Ah*-responsive and *Ah*-nonresponsive animals, results in marked individual variation in the induction of P450 1A1 and other enzymes.

The transcriptional regulation via the *Ah* receptor is clearly not the only induction-mechanism, because several compounds (e.g. phenolic antioxidants) are known to induce glutathione S-transferase without the induction of P450 1A1 (Rushmore *et al*, 1990). Moreover, induction of glucuronyl transferase and DT-diaphorase are both also described to occur without co-induction of P450 1A1 (Burchell *et al*, 1987; Prochaska *et al*, 1985), indicating other regulatory mechanisms as well.

Phenobarbital-inducible genes and clofibrate-inducible genes

Treatment of rats with phenobarbital increases the expression of many genes including those coding for P450 2B1 and 2B2 (Atchinson and Adesnik, 1983), glucuronyl transferase (Bock *et al*, 1987), epoxide hydrolase and DT-diaphorase (Hardwick *et al*, 1983). To date, the mechanism of induction of the P450 2B family is still unknown.

Clofibrate also induces a battery of enzymes via transcriptional activation. The enzymes include P450 4A1 (Hardwick *et al*, 1987) and fatty acyl-CoA oxidase (Reddy *et al*, 1986). A receptor for clofibrate has been purified (Lalwani *et al*, 1987; Isseman and Green, 1990), but the exact regulatory mechanism has not been elucidated yet.

Steroid-inducible genes

Rat P450 3A1 proteins are induced by glucocorticoids, such as dexamethasone and pregnenolone 16α-carbonitrile. The induction is primarily an activation of gene transcription, but mRNA stabilization has also been reported (see 4.2.). It has been suggested that the glucocorticoid receptor which controls a variety of hepatic genes (e.g. the gene encoding tyrosine aminotransferase) is involved. The induction of P450 3A1, however, requires a 10-fold higher level of steroid than is needed to induce tyrosine aminotransferase (Schuetz *et al*, 1984). Moreover, certain steroids do not co-induce both P450 3A and tyrosine aminotransferase (Schuetz and Guzelian, 1984), indicating a different mechanism for the induction of P450 3A enzymes.

Phenobarbital induces two proteins in the P450 3A family: the constitutively expressed 3A2, but also 3A1 which is not expressed in untreated rats. The fact that only P450 3A1 is induced by steroids, suggests that there are different mechanisms involved for these different classes of inducers.

Constitutive P450 genes

During development several biotransformation enzymes are differentially expressed in the rat. Immediately after birth the protein levels of P450 2E and 2D are markedly enhanced (Song *et al*, 1986), whereas at the onset of puberty P450 2C6 and 2C7 are elevated (Waxman *et al*, 1985). The mechanism by which these genes are activated is not fully understood. Blood testosterone levels appear not to regulate these forms (Bandiera *et al*, 1986; Dannan *et al*, 1986).

On the other hand the male-specific P450 2C11 and the female-specific P450 2C12 are both responsive to physiological signals triggered during the period of sexual maturation. Both testosterone and growth hormone influence the regulation of these sex-specific P450 proteins. Testosterone does not act directly on the liver, but rather affects the pulsatile secretion of growth hormone by the pituitary. Adult male rats show a pulsatile secretions of pituitary growth hormone, while adult females have more constant levels. Hypophysectomy reduces the expression of P450 2C11 or 2C12 in males and females, respectively. Periodic injections of growth hormone in males and continuous infusion of growth hormones in females reestablish the levels of these sex-specific P450s (Mode *et al*, 1989).

Growth hormone also plays a role in the suppression of some P450s. This explains the decline of several P450s (P450 2A, 2E, and 3A) during development, while these forms are at relatively high levels in the immature male and female rat (Schenkman *et al*, 1989). Moreover, P450 2B1 and 2B2 have been reported to be suppressed in both male and female rats by growth hormone (Yamazoe *et al*, 1987, Schuetz *et al*, 1990).

4.2.2. Posttranscriptional regulation

In addition to regulation of gene expression, several P450 forms are also regulated posttranscriptionally. Stabilization of P450 mRNA is reported for P450 1A2 (Gonzalez *et al*, 1984) and 3A2 (Watkins *et al.* 1987). The most profound example of posttranscriptional control, however, is the regulation of the P450 2E1 enzyme (Yang *et al*, 1990). This enzyme is induced under a variety of conditions, e.g. ethanol treatment, fasting and chemically-induced diabetes, without enhanced transcription of the 2E1 gene. Several studies suggest that the binding of inducers to 2E1 results in a protection of the enzyme against degradation. Furthermore, fasting causes an increase in 2E1 mRNA and protein. In this regard, it has been suggested that intracellular levels of acetone or other ketone bodies may play an important role in the regulation of P450 2E1 under normal physiological conditions (Miller and Yang, 1984).

5. Consequences of induction of biotransformation enzymes

Induction is generally considered as an adaptive response to the inducer itself often leading to increased elimination and detoxication of the inducer and other chemically related compounds (Breckenridge, 1987). However, induction may be a two-edged sword which sometimes can also lead to increased susceptibility to toxic compounds. Many biotransformation enzymes, in particular certain P450s, may generate chemically-reactive intermediates from relatively unreactive parent compounds. The balance between the bioactivation and inactivation routes can be markedly disturbed after exposure to inducing agents. This balance should not be simplified and divided between phase I and phase II enzymes only. Even among the P450s, substances can be detoxified by one form, while it is bioactivated by another. For example, the potent hepatocarcinogen aflatoxin B_1 is converted by P450 2C11 and 1A2 to the highly reactive intermediate, aflatoxin B_1-8,9-epoxide. The epoxide binds covalently to DNA, thereby causing an initiating event in carcinogenesis. In contrast, P450 1A1 convert aflatoxin B_1 primarily to aflatoxin M_1, a much less potent carcinogen than the parent compound. Table 6 includes some carcinogens of which metabolism by individual P450s have been studied in more detail.

A major problem associated with these studies is the estimation of the fraction of total activity catalysed by a particular form of P450. Most studies involve inhibitors (antibodies, (non)competitive inhibitors), which are often not as selective as one would hope. Another problem is that the highly inducible P450s may not be relevant to bioactivation of procarcinogens in untreated animals (Guengerich, 1988).

Table 6: Activation and inactivation of precarcinogens by rat P450-enzymes.

Precarcinogen	P450s involved in bioactivation [a]	references
2-acetylaminofluorene	2B1, 1A2	Holme *et al*, 1986
aflatoxin B_1	2C11, 1A2	Ishii *et al*, 1986; Ramsdell and Eaton, 1990;
benzo[*a*]pyrene	1A1, 2B1	Robertson *et al*, 1983
7,12-dimethylbenz[*a*]-anthracene	2C6/11	Morrison *et al*, 1991
N,N-dimethylnitrosamine	2E1	Yang *et al*, 1990
IQ(2-amino-3-methylimidazo [4,5-*f*]quinoline	1A1, 1A2	Yamazoe *et al*, 1984
3-methylcholanthrene	1A1	Shimada and Nakamura, 1987
2-naphthylamine	1A2	Hammons *et al*, 1985

[a] In some cases only a limited number of P450 enzymes have been studied. Adapted from Guengerich *et al*, 1988).

The advanced knowledge of the dual role of cytochrome P450s emphasize to study the effects of several, in particular daily ingested compounds (food compounds and persistent industrial chemicals) on the catalytic activities of selective cytochrome P450 and phase II enzymes. In this respect, one group of food compounds known to affect both biotransformation enzyme activities as well as the risk of carcinogenesis are glucosinolates in cruciferous vegetables.

6. Enzyme-inducing capacity of cruciferous vegetables

Cruciferous vegetables (cabbage, cauliflower, Brussels sprouts, broccoli) contain appreciable amounts of glucosinolates, which upon hydrolysis yield various substances, e.g. indoles, isothiocyanates, nitriles, thiocyanates, and goitrins (Fenwick and Heaney, 1983). Several studies reported the adverse effects on thyroid function following high consumption of cruciferous vegetables. In this respect, thiocyanates and goitrins have been shown to be goitrogenic, due to both inhibition of the production of thyroid hormone, as well as direct interference with the synthesis of thyroxine (Fenwick *et al*, 1983). Only part of these effects can be reversed by dietary iodine supplementation. In a recent clinical study, however, no goitrogenic effects could be observed after daily consumption of progoitrin rich Brussels sprouts (McMillan *et al*, 1986).

Apart from the possible effects on thyroid function, cruciferous vegetables are known to affect the carcinogenic process. Epidemiological evidence (Manousos *et al*, 1971; Graham *et al*, 1978; Haenszel *et al*, 1980; Graham, 1983) suggested that a frequent consumption of cruciferous vegetables was associated with a decreased risk of tumour formation in the gastro-intestinal tract in humans. In 1982 the National Research Council

in its publication, *Diet, Nutrition and Cancer* (1982) recommended increased consumption of cruciferous vegetables in an effort to decrease the risk of cancer in human populations. The exact mechanism of the protective effect of these vegetables, however, was unknown. One likely explanation is a change in the metabolism of procarcinogens. Several compounds in cruciferous vegetables are known to induce biotransformation activities when fed to rats, i.e. glucosinolates (Chang and Bjeldanes, 1985; McDanell *et al*, 1988), but also flavonoids (Vernet and Siess, 1986) and dithiolthiones (Ansher *et al*, 1986).

The levels of glucosinolates in different cruciferous vegetables vary widely depending on species, variety, cultivation conditions etc. (Table 7). The mean daily intake of total glucosinolates in the United Kingdom has been estimated to be *ca* 46 and 29 mg/person from fresh and cooked sources, respectively (Sones *et al*, 1984). The mean daily intake of glucobrassicin (indolylmethyl glucosinolate) herein has been estimated to be 12.5 mg/person (from fresh sources) and 7 mg/person from cooked sources.

Table 7. Levels of individual glucosinolates in cruciferous vegetables

	Glucosinolate content (mg/100 g fresh weight)		
Glucosinolate	Cabbage	Cauliflower	Brussels sprouts
sinigrin	7.4 - 64.6	0.5 - 62.7	1.6 - 111.4
gluconapin	0.0 - 5.3	0.0 - 2.8	2.5 - 90.9
progoitrin	0.8 - 12.6	0.0 - 10.1	12.5 - 129.6
glucoiberin	2.3 -129.0	0.6 - 41.9	0.0 - 71.1
glucobrassicin	4.5 - 97.1	6.6 - 78.9	26.4 - 158.6
neoglucobrassicin	0.0 - 10.1	0.6 - 16.5	1.0 - 17.7

(From Sones et al, 1984).

The metabolites of indolylmethyl glucosinolate (i.e. indole-3-carbinol, indole-3-acetonitrile and 3,3'-diindolylmethane) are known to be the most potent inducers of monooxygenase activities known so far (for review see McDanell *et al*, 1988; Fig. 3). The metabolites are formed during storage and processing of the vegetables by the action of the plant enzyme myrosinase (thioglucoside glucohydrolase E.C. 3.2.3.1).

In rats, but also in man and mice, feeding a diet containing either cruciferous vegetables or the isolated constituent, indole-3-carbinol, results in changes in biotransformation enzyme activities. Moreover, in several of these studies using laboratory animals a decreased chemically induced-tumorigenesis was observed in several organs (Wattenberg and Loub, 1978; Boyd *et al*, 1982; Wattenberg *et al*, 1986). On the other hand, some studies reported enhanced tumorigenesis e.g. when cabbage or indole-3-carbinol was fed after the pro-carcinogen (Srisangnam *et al*, 1980; Pence *et al*, 1985; Birt

et al, 1987). Despite the large amount of studies on changes in biotransformation activities, little knowledge is available of the effects of cruciferous vegetables and indole-3-carbinol on specific cytochrome P450s in liver and small intestine.

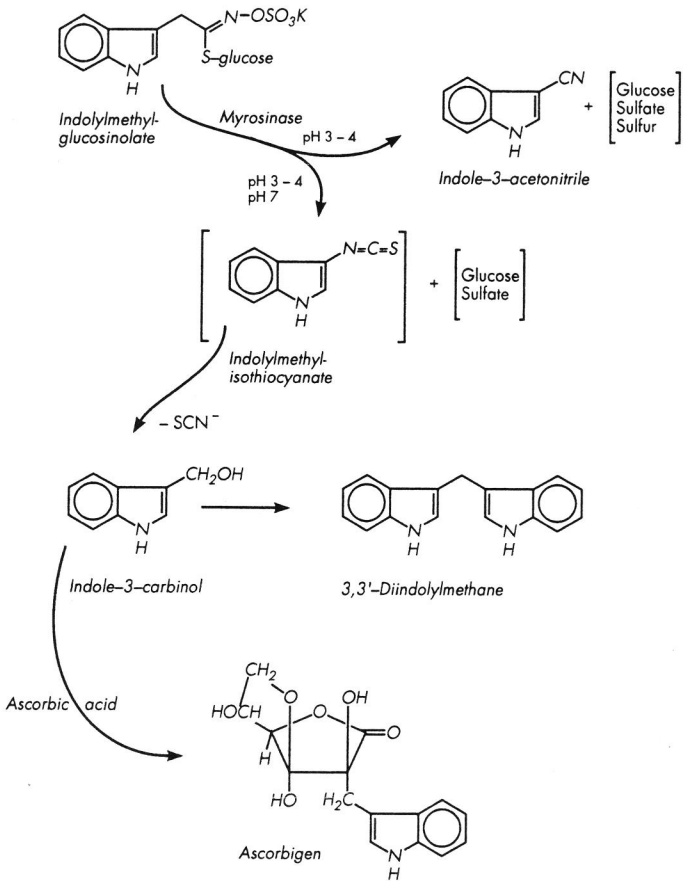

Fig. 3. Breakdown of indolylmethyl glucosinolate by myrosinase (from McDanell *et al*, 1988).

7. *In vitro* models: primary cultures of hepatocytes

Most bioassays concerning induction studies involve *in vivo* exposure of laboratory animals (mainly rodents) to the specific chemical. Per chemical and concentration a minimal of three animals is needed to test the effects on biotransformation activities. For reasons of reduction in the number of animals, refinement of the bioassay and replacement of *in vivo* studies, *in vitro* models with primary cultures of hepatocytes may offer a good alternative for at least part of this animal studies.

The applicability of hepatocytes for pharmacological and toxicological studies has been reviewed by Moldeus *et al* (1987), Guillouzo (1986), Chenery (1988) and Paine (1990). Primary hepatocyte cultures as an *in vitro* model has several advantages and disadvantages. Cells from one liver can be divided over a large number of experimental units, which can be used for studying many different parameters all with one homologous batch of cells. The culture conditions of the cells are well defined and can be changed dependent of the studied problem. In comparison with cellular fractions like microsomes, the intact cell retains many biological system factors: uptake and intracellular distribution of the substrate; generation of cofactors and cosubstrates; interrelationship between metabolic pathways involved in drug metabolism; and competition between endogenous and exogenous substrates (Moldeus, 1987).

However, a major problem of hepatocytes is the rapid loss of metabolic activity during culture, especially those of cytochrome P450. Many researchers have tried to prevent this loss by the use of media supplemented with either certain hormones (Decad *et al*, 1977) or ligands such as metyrapone (Paine, 1990), or culturing hepatocytes in a co-culture with epithelial cells (Guillouzo, 1986) or on extracellular membranes (Schuetz *et al*, 1988). Although some were partly successful to stabilize the total cytochrome P450 level, selective changes were observed in the pattern of the different P450 enzymes during culture. One of the consequences of the rapid loss of cytochrome P450 and its associated catalytic activities is the high demand on the sensitivity and detection limits of several biochemical determinations, when using only a limited amount of cells.

For many years cultured hepatocytes are widely used as a model system in *in vitro* toxicology for the study of e.g. the role of biotransformation in the toxicity of a compound. The cells can be obtained from a number of species, including rat, hamster, rabbit, chicken, etc. There is also limited experience with human liver cells in culture. The use of hepatocytes obtained from different species may lead to a better understanding of of species differences in the toxicity of xenobiotics, thus improving interspecies extrapolation (Blaauboer *et al*, 1990).

In vitro test systems can be divided in three different types: i.e. screening test, adjunct tests and replacement tests (Balls *et al*, 1990). **Screening tests** are defined to be simple, rapid and inexpensive for use in making preliminary decisions or in setting priorities among large groups of chemicals for selection of further testing, either with intact animals or more sophisticated *in vitro* tests. **Adjunct tests** are tests conducted in conjunction with animal tests to evaluate toxicity, to elucidate mechanisms of toxicity or to further investigate specific observations without the need to perform additional animal

studies. **Replacement tests** are tests developed with the intent of replacing existing *in vivo* or *in vitro* tests, in all respects, including regulatory acceptance.

Due to the loss of some physiological functions with culture time, such as the decline of biotransformation activities, hepatocyte cultures to date are restricted in their use as a full replacement for *in vivo* situation. On the other hand, several studies indicate that primary cultures of hepatocytes retain their capacity to respond to some P450 inducers by increasing the synthesis of specific mRNAs and proteins and by enhancing associated activities (Elshourbagy *et al*, 1981; Newman and Guzelian, 1982; Steward *et al*, 1985; Bars *et al*, 1989), although the mechanism of gene expression may be on a different level *in vitro* compared to *in vivo* (Silver and Krauter, 1988; Pasco *et al*, 1988). In many of these studies the researchers were focused on the induction of one or a limited number of P450s. Advanced knowledge on the specificity of individual P450 forms towards substrates, now enhances the possibility to determine the P450 profile in more detail.

8. Scope of this thesis

The investigations described in this thesis are primarily concerned with the application of primary cultures of hepatocytes in the determination of the induction potential of cytochrome P450 enzymes. The main objective herein was not to elucidate the mechanism of induction at a molecular level, but to study the changes in P450 dependent activities.

In the first part of the study, methods were developed to determine several P450-dependent activities directly in intact hepatocytes (chapter 2) and the effects of model-inducers on changes in P450-dependent activities in cultured rat hepatocytes (*in vitro*) were compared to the changes in the liver from living animals (*in vivo*) treated with the same model inducers (chapter 3).

In the second part (chapter 4, 5, 6 and 7) both *in vivo* and *in vitro* studies are complementarily used to study the enzyme-inducing capacity of Brussels sprouts and more specifically some breakdown products of indole-glucosinolates. Two *in vivo* studies were performed to study time- and dose-related effects of Brussels sprouts and indole-3-carbinol on specific cytochrome P450s as well as phase II enzymes in liver and small intestine of the rat (Chapter 4 and 5). To elucidate which indole metabolite might be responsible for the changes in biotransformation enzymes, *in vitro* studies were used complementarily to these *in vivo* experiments. Regarding the difference in effects of indole-3-carbinol *in vivo* with *in vitro*, special attention was paid to the formation and identification of acid condensation products formed out of indole-3-carbinol in the acid environment of the stomach (chapter 6). Finally, the ability of these compounds to affect several biotransformation enzymes was studied *in vitro* using both rat and monkey hepatocytes (chapter 7). In chapter 8 the results presented in this thesis are discussed.

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CHAPTER 2

THE ISOENZYME PATTERN OF CYTOCHROME P450 IN RAT HEPATOCYTES IN PRIMARY CULTURE, COMPARING DIFFERENT ENZYME ACTIVITIES IN MICROSOMAL INCUBATIONS AND IN INTACT MONOLAYERS

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Abstract

Changes in the isoenzyme pattern of cytochrome P450 during culture were investigated in primary cultures of rat hepatocytes, measuring specific enzyme activities in microsomes prepared from cultured cells as well as in intact monolayers. Assays of 7-ethoxyresorufin O-deethylation (EROD), 7-pentoxyresorufin O-depentylation (PROD), aniline 4-hydroxylation (AH) and the specific regioselective hydroxylation of testosterone were used as representatives of the activities of seven isoenzymes of cytochrome P450. The isoenzyme profile expressed as catalytic activities was qualitatively and quantitatively similar in microsomes obtained from freshly isolated hepatocytes in comparison with microsomes obtained from whole livers of untreated rats. There was a relatively high activity in EROD, AH and the oxidation of testosterone at the 7α, 2α, 6β, 16α and 17 sites (androstenedione). During culture, these microsomal enzyme activities declined at a similar rate to ca. 50% of the activities of microsomes prepared from freshly isolated hepatocytes after 24 hr and to 15% after 96 hr. The overall decline of cytochrome P450-dependent activities during culture was not accompanied with gross changes in catalytic profile. Determining the same drug-metabolizing activities directly in intact hepatocyte monolayers revealed a much higher metabolic rate for all measured P450-dependent activities. The profile of the catalytic activities was essentially the same as measured in microsomes prepared from cultured hepatocytes. The relatively low activity towards the 7α site of testosterone measured in intact hepatocytes, however, remained constant during culture. Determination of enzyme activities directly in intact hepatocytes is a convenient way of studying changes in monooxygenase activities of different P450 isoenzymes *in vitro*.

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Introduction

A wide variety of foreign compounds are metabolized by the hepatic biotransformation system. The activity of this system can be altered by many factors, including foreign chemicals. A pivotal role in biotransformation is played by cytochrome P450, a multigenic family of haemoproteins which determine the duration of action of many drugs and play a key role in chemical carcinogenesis and toxicity [1,2]. The isoenzymes differ from each other in substrate specificity and catalytic activity [3]. Detailed description of the isoenzyme pattern of cytochrome P450 is desirable to provide a basis for studies on the monooxygenase-dependent metabolism and the subsequent effects of these biotransformation changes on biological effects of various xenobiotics.

Primary hepatocyte cultures represent a potentially useful model for studies on the effects of induction and different isoenzyme patterns of cytochrome P450 in correlation with toxicity of various compounds [4]. It is well known that the content and catalytic activities of cytochromes P450 decline during culture (see rev. [5]). Several attempts have been made to maintain either the content of cytochrome P450, the associated catalytic activities, or both [5-10]. It is not clear whether the higher P450 levels and/or higher catalytic activities determined in these studies are the result of either stabilization or a more or less selective induction of one or more cytochrome P450 isoenzymes.

Monitoring specific biotransformation activities in addition to immunoblotting is a reliable way of describing changes in the population of cytochrome P450 isoenzymes [11,12]. Burke et al. [13] investigated the selective dealkylation of phenoxazone alkyl derivatives, 7-ethoxyresorufin and 7-pentoxyresorufin, by two specific isoenzymes, viz. P450 1A1 (EROD) and P450 2B1 (PROD). Aniline is 4-hydroxylated mainly by P450 2E1 and P450 1A2 [14]. Furthermore, it has been shown that hydroxylation of testosterone is catalysed by different isoenzymes of cytochrome P450 with a high degree of regio- and stereoselectivity. Testosterone 7α-hydroxylation activity reflects the levels of P450 1A1 [15], 16β-hydroxylation activity the levels of P450 2B1, 2α-hydroxylation activity the levels of P450 2C11, and the formation of 6ß-, 15β- and 18-hydroxy-testosterone reflects the activity of P450 3A [16,17,36].

In the present study we used different techniques to describe the changes in monooxygenase activity of P450 isoenzymes in primary rat hepatocytes during culture. Various specific activities were measured in microsomes prepared from primary cultured hepatocytes. Furthermore, to maintain the interrelationship between metabolic pathways in the intact cell [4,18] and to avoid a possible selective destruction of isoenzymes during microsomal preparation from hepatocytes [19], we developed a more convenient way of determining catalytic activities directly in intact hepatocyte monolayers. The profiles of P450-dependent activities in intact cells as well as in microsomes from cultured cells are compared with the *in vivo* pattern as determined in microsomes prepared from whole liver.

Materials and methods

Materials

Newborn calf serum was purchased from Gibco Europe, Breda, The Netherlands. Testosterone, androstenedione, 11β- and 16α-hydroxytestosterone (11β- and 16α-OHT), Williams' medium E, insulin and hydrocortisone were obtained from Sigma Chemical Co., St Louis, MO, USA. 2α-OHT was a kind gift from Prof D.N. Kirk (Queen Mary College, University of London), 15β- and 12β-OHT were gifts from G.D. Searle and Co., Skokie, IL, USA. 6β-, 7α-, 16β- and 19-OHT were obtained from Steraloids, Wilton, NH, USA. β-Glucuronidase/arylsulphatase (E.C. 3.2.1.31/ E.C. 3.1.6.1) was supplied by Boehringer (Mannheim, FRG). All other chemicals were of analytical grade. The antibodies used in this study were all kind gifts. Monoclonal antibodies against P450 1A1/2, P450 2B1/2, P450 3A were from Dr P.J. Kremers, Université de Liège, Belgium; a polyclonal antibody against P450 2E1 was from Dr I. Johansson, Karolinska Institutet, Stockholm, Sweden; a polyclonal antibody against P450 4A1 was from Dr G.G. Gibson, University of Surrey, Guildford, UK.

Animals

Male Wistar rats (Riv.Tox(M)), RIVM, Bilthoven, The Netherlands), weighing 180 - 250 g, were fed *ad libitum* a TNO-Institute grain-based open-formula diet (TNO-Toxicology and Nutrition Institute, Zeist, The Netherlands) and had free access to drinking water.

Cell isolation and culture

Rat hepatocytes were isolated using the two-step collagenase perfusion technique described by Seglen [20] as modified by Paine *et al.* [21]. The cells were plated on 9 cm tissue culture dishes (Sterilin) at a density of 8×10^6 cells/dish in 10 ml Williams' medium E supplemented with 3% (v/v) newborn calf serum, 1 μM insulin, 10 μM hydrocortisone and 50 mg/l gentamycin. Cells were incubated in a humidified atmosphere of air (95%) and CO_2 (5%) at 37°C. After 4 hr in culture, media were replaced. Thereafter, media were refreshed every 24 hr.

Preparation of microsomes

Whole livers: Livers from control animals were perfused with ice-cold saline and homogenized in 0.15 M KCl containing 0.1 mM EDTA using a Potter-Elvehjem glass-teflon homogenizer. Microsomes were prepared by centrifugation (2 x 20 min, 9000 *g*; supernatant 60 min, 105,000 *g*). The microsomal pellet was resuspended in Na-phosphate buffer (0.1 M, pH 7.8) containing 0.1 mM EDTA, frozen quickly in liquid N_2 and stored at -70°C.

Cultured hepatocytes: Per treatment group 7 culture dishes were washed in ice-cold PBS and scraped with a rubber policeman in 1 ml PBS/dish. Cells were centrifuged (3 min, 50 *g*); supernatant was replaced by 2.5 ml 0.15 M KCl containing 0.1 mM EDTA and cells were kept at -70°C until preparation of microsomes. Cells were homogenized on ice by sonication using an MSE 100 W ultrasonic disintegrator.

Sonication for 20 sec appeared to be optimal. After centrifugation for 2 x 20 min at 9000 ***g*** the supernatant was centrifuged for 60 min at 105,000 ***g***. The microsomal pellet was resuspended by sonication (20 sec) in 700 µl 0.1 M Na-phosphate buffer (pH 7.8) containing 0.1 mM EDTA on ice, frozen quickly in liquid N_2 and stored at -70°C. One mg cellular protein yielded *ca.* 75 µg microsomal protein.

Enzyme assays

Cytochrome P450 and protein

Contents of protein and total cytochrome P450 were determined according to Rutten *et al.* [22] using a double-beam spectrophotometer (Pye Unicam 8800). Total amount of cytochrome P450 could not be determined in homogenized hepatocytes of 72 and 96 hr in culture because of the very low amount of cytochrome P450 in combination with the high levels of turbidity.

Ethoxy- and pentoxyresorufin O-dealkylation (EROD and PROD)

Fluorometric determinations of liver and hepatocyte microsomal 7-ethoxyresorufin O-deethylation (EROD) and 7-pentoxyresorufin O-dependentylation (PROD) activities were performed according to the method of Burke *et al.* [23] using a Cobas-Bio centrifugal analyzer, equipped with a spectrofluorimeter. Less than 200 µg microsomal protein was used in the incubation mixture of 0.1 M phosphate buffer (pH 7.4) with a final volume of 320 µl. Substrate was added in diluted DMSO (final DMSO concentration 0.1% v/v). Substrate concentrations of 5 µM were used.

Determinations directly in intact hepatocytes were as follows. Hepatocyte monolayers were washed twice with Hanks' balanced salt solution (HBSS) gassed with carbogen (95% O_2 /5% CO_2 v/v). The incubation was initiated by adding 4 ml HBSS (37°C) containing 5 µM substrate and 10 µM dicumarol. Dicumarol was added during incubation to prevent further metabolism of resorufin by the cytosolic enzyme DT-diaphorase [24]. To determine linearity of enzyme activity medium samples of 1 ml each were taken every 5 min during a period of 15 min (EROD) or 20 min (PROD). After dilution (1:1) of the samples with incubation mixture fluorescence was determined using a Kontron SFM 25 fluorometer. The fluorescence of resorufin appeared to be pH-dependent. A stable fluorescence was obtained after addition of 100 µl 0.1 M NaOH to the 2 ml diluted medium samples to bring the pH above 7.5.

Aniline 4-hydroxylation (AH)

The AH activity of liver and hepatocyte microsomes was determined according to Chhabra *et al.* [25], using an aniline concentration of 15 mM. The reaction was stopped after 45 min at 37°C, by adding 0.5 ml 20% TCA on ice. After centrifugation (5 min, 100 ***g***), the formation of 4-aminophenol was determined spectrophotometrically at 630 nm, according to Imai *et al.* [26].

Determination of aniline 4-hydroxylation in intact hepatocytes using a short incubation time was not possible. The low activity of this reaction resulted in amounts of products below the limit of detection when incubation times comparable with the other measured reactions were used [27].

Testosterone hydroxylation

Determinations in microsomal preparations were carried out in 1 ml incubation mixture containing K-phosphate buffer (50 mM, pH 7.4), $MgCl_2$ (3 mM), EDTA (1 mM), $NADP^+$ (1 mM), glucose 6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (1 unit/ml), testosterone (250 µM) and 300-500 µg microsomal protein. Testosterone was added as methanolic solution (final methanol concentration 2% v/v). Incubation mixture and microsomes were mixed while standing on ice. Reactions were started by heating the mixture in a 37°C water bath and stopped after 15 min by addition of 6 ml ice-cold dichloromethane.

For determinations in intact hepatocytes, monolayers were washed twice with HBSS gassed with carbogen. The reaction was started by adding HBSS (37°C) containing 250 µM testosterone (added as methanolic solution, final concentration methanol 0.5% v/v) and stopped after 15 min by separating the medium and cooling it on ice. Cells were scraped in ice-cold PBS and both cells and medium were stored at -20°C until extraction.

After extraction of the reaction products present in 1 ml medium or cell suspension with 6 ml dichloromethane, the aqueous phase containing the precipitated protein was removed and dichloromethane was evaporated under a stream of N_2 at room temperature. The residue was dissolved in 130 µl 50% (v/v) methanol/water.

HPLC analysis of testosterone metabolites

Testosterone metabolites were analysed according to Funae and Imaoka [28]. Analyses were performed on an HPLC system consisting of an LKB 2150 HPLC pump, a Kontron MSI 660 autosampler equipped with a 20 µl injection loop, an LKB 2151 variable-wavelength monitor and a Pye Unicam LC-XP gradient programmer. Testosterone oxidation products were separated using a Chromsep C18 (200 x 3 mm i.d., particle size 5 µm) reversed phase column preceded by a 10 mm C18 guard column (Chrompack, Middelburg, the Netherlands). The OHTs were eluted with aqueous solutions of 25% methanol from 0 to 10 min followed by a linear gradient to 40% methanol and 3.5% acetonitrile from 10 to 45 min, at a flow rate of 0.8 ml/min. Column temperature was kept at 50°C. Column effluents were monitored at 254 nm. Metabolites were quantified by comparing their peak areas with those of authentic standards, using a Shimadzu C-R1A Chromatopac integrator. 11β-OHT was used as internal standard [29].

Western immunoblotting

Separation of microsomal proteins was carried out on a Biorad mini Protean II cell using the sodium dodecyl sulphate polyacrylamide (SDS-PAGE) discontinuous system of Laemmli [30]. The resolved proteins were electrophoretically transferred to polyvinylidenedifluoride (PVDF) sheets (Millipore) according to the method of Towbin *et al.* [31] and immunochemically stained using antibodies to specific P450 forms.

Results

Profile of cytochrome P450-dependent activities in microsomes prepared from cultured hepatocytes

To define optimal conditions for microsome preparation from hepatocytes different sonication times were used to homogenize the cells. The amounts of microsomal protein and cytochrome P450 recovered after 2 - 30 sec of sonication are shown in Table 1. No degradation of cytochrome P450 in the form of cytochrome P420 was detected in the spectral measurements. Longer sonication resulted in higher amounts of microsomal protein and cytochrome P450. In all further experiments a sonication time of 20 sec was used, because this time yielded the highest EROD and PROD activity per mg microsomal protein, although the PROD activity per nmol P450 was somewhat less at that time. Recovery of cytochrome P450 in microsomes was ca. 30% as compared to the amount of cytochrome P450 in homogenized hepatocytes (ca. 14.3 and 48 pmol P450 per one million hepatocytes in microsomes and cell homogenates, respectively). Most of the cytochrome P450 got lost in the first 9000 *g* pellet, probably due to incomplete cell disruption.

Table 1. The effect of sonication time during preparation of microsomes from freshly isolated hepatocytes on the recovery of microsomal protein, cytochrome P450 and two cytochrome P450-dependent catalytic activities.

sonication time (s)	protein (mg/ml)	P450 (pmol/mg protein)	EROD		PROD	
			(pmol/min. mg protein)	(pmol/min. nmol P450)	(pmol/min. mg protein)	(pmol/min. nmol P450)
2	3.70	186.39	37.44	200.86	5.40	28.97
5	4.19	216.06	55.56	257.15	6.56	30.36
10	4.44	235.16	51.54	219.17	6.40	27.22
20	5.67	275.72	62.40	226.32	6.62	24.01
30	6.62	294.52	60.96	206.98	5.96	20.24

Data are means of duplicate preparations.

The content of cytochrome P450 and the different catalytic activities in microsomes of freshly isolated hepatocytes were similar to those in microsomal preparations from whole livers of control animals (Fig. 1a-b). There was a relatively high activity towards aniline, a relatively low activity towards 7-ethoxyresorufin, and a negligible activity towards 7-pentoxyresorufin. The major oxidation products formed after testosterone exposure were 16α-OHT, androstenedione and 2α-OHT, followed by 6β-OHT and 7α-OHT. The formation of 15β-OHT, 16β-OHT, and an unknown metabolite with a relative retention time of 0.83 (probably 18-OHT [16]) was negligible.

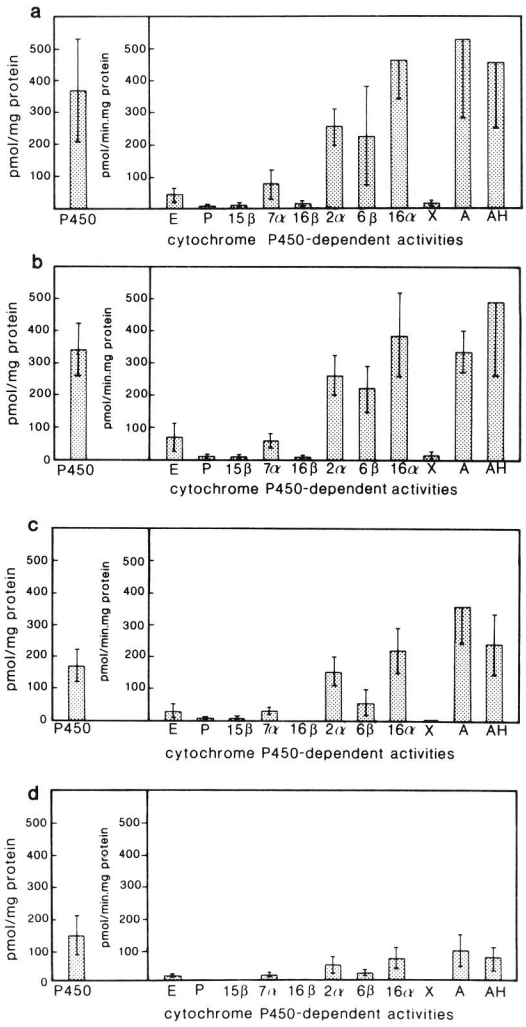

Fig. 1. Profiles of specific and less specific cytochrome P450-dependent activities in microsomes prepared from whole livers of control animals (a), from freshly isolated cells (b), and from cultured cells after 24 h (c) and 96 h in culture (d). P450, total cytochrome P450 content; E, 7-ethoxy-resorufin O-deethylation; P, 7-pentoxyresorufin O-depentylation; 15β, the formation of 15β-OHT; 7α, 7α-OHT; 16β, 16β-OHT; 2α, 2α-OHT; 6β, 6β-OHT; 16α, 16α-OHT; X, an unknown metabolite with Rf= 0.83; A, androstenedione; AH, aniline 4-hydroxylation. Values represent means ± SD, n = 5 rats *in vivo*, n = 10 rats *in vitro*.

Table 2. Different cytochrome P450-dependent activities expressed as percentage of the summation of measured activity in microsomes prepared from primary hepatocytes during culture.

time in culture (hr)	total P450 (pmol/mg protein)	percentage of the sum of measured activity											sum of measured activity (pmol/min.mg protein
		EROD	PROD	15ß	7α	16ß	2α	6ß	16α	X	A	AH	
0	343 ± 84	3.6	0.4	0.3	2.8	0.2	14.6	11.8	21.5	0.9	18.5	25.4	1842
24	170 ± 53	2.5	0.5	0.1	2.5	0.1	13.9	5.4	20.3	0.3	33.1	21.4	1091
48	176 ± 46	1.3	0.1	0.1	2.9	0.0	13.8	4.1	21.7	0.1	32.4	23.5	838
72	151 ± 46	1.8	0.1	0.2	3.3	0.0	14.5	4.7	22.5	0.0	30.8	22.0	520
96	136 ± 65	2.5	0.1	0.2	3.7	0.0	14.3	6.3	22.5	0.0	30.2	20.1	312

Data are means of n=10. For abbreviations see legend Fig. 1.

Table 3. Different cytochrome P450-dependent activities as percentage of the summation of measured activity, expressed per mg cellular protein in intact monolayers of hepatocytes during culture.

time in culture (hr)	percentage of the sum of measured activity										sum of measured activity (pmol/min.mg protein)
	EROD	PROD	15β	7α	16β	2α	6β	16α	X	A	
4	0.3	0.5	1.3	1.0	1.5	7.3	30.2	14.1	2.2	41.7	1349
24	0.7	1.9	1.2	1.3	1.2	8.3	16.5	16.1	1.5	51.2	963
96	0.7	1.0	1.4	1.8	0.4	5.6	7.7	12.3	1.0	68.0	529

Data are means of n=3. For abbreviations see legend to Fig. 1.

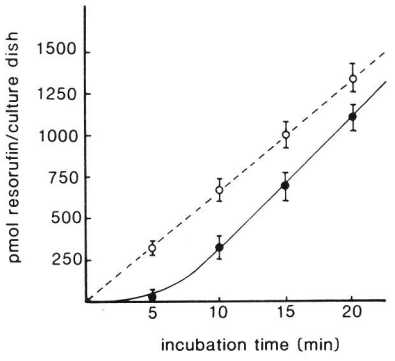

Fig. 2. Production of resorufin during EROD and PROD assay in monolayers of hepatocytes. ○ , EROD; ● , PROD; values are means ± SD, each curve representing four different 24 hr old hepatocyte monolayers.

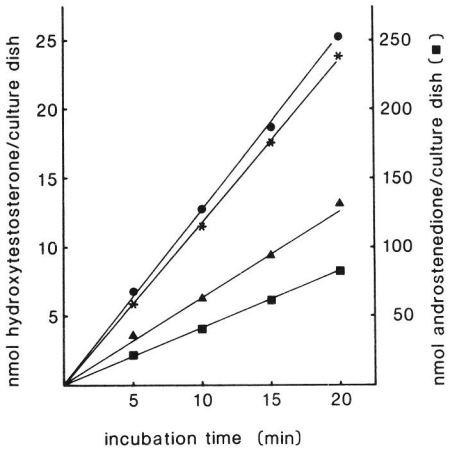

Fig. 3. Production of different metabolites of testosterone in monolayers of hepatocytes. ✱ , 6ß-OHT; ● , 16α-OHT; ▲, 2α-OHT; ■ , androstenedione; values are means of duplicate 24 hr old hepatocyte monolayers.

In immunochemical studies no P450 1A1, P450 2B1/2 and P450 4A1, and only minor amounts of P450IA2, P450 2C11, P450 2E1 and P450 3A could be detected (results not shown). These results show that the procedures for preparation of microsomes from freshly isolated hepatocytes do not alter the P450 isoenzyme profile.

During culture AH activity and the formation of 2α-OHT, 7α-OHT and 16α-OHT declined similarly, showing an average activity of 55% of those measured in freshly isolated hepatocytes after 24 hr, and 18% after 96 hr in culture. A more rapid decline (to ca. 25% of freshly isolated cells) was measured for EROD activity and the formation of 6β-OHT during the first 24 hr in culture. The low levels of apoprotein of P450IA2 and P450 3A in freshly isolated hepatocytes diminished during culture. After 24 hr in culture, the distribution of the measured catalytic activities did not change significantly (Table 2).

Optimal conditions for determination of cytochrome P450-dependent activities in hepatocyte monolayers

For determining the EROD and PROD activities in intact hepatocyte monolayers a substrate concentration of 5 μM appeared to be optimal (data not shown). EROD activity showed no lag time, while PROD activity revealed a lag time of 7 min. After that time the formation of resorufin was linear for at least 10 min (Fig. 2).

Formation of different hydroxy metabolites of testosterone in intact hepatocytes was linear over a 20 min incubation time (Fig. 3). No difference could be detected in the amount of testosterone metabolites in the media with or without deconjugation (data not shown). Testosterone itself was glucuronidated, but only to a small extent (less than 10%). Therefore, no deconjugation of the media samples had to be performed.

Extraction of reaction products of testosterone from both cells and media after a 15 min incubation period revealed that testosterone metabolites retained within the cells amounted to 9 to 18% of the content in the medium for all metabolites. Therefore, the metabolite pattern in the medium could be regarded as representative of the pattern in the complete incubation. Representative chromatograms of an extract of an monolayer incubation and of an microsomal incubation, both of hepatocytes 24 hr old, are presented in Fig. 4a-b. A mixture of authentic standards is shown in Fig. 4c.

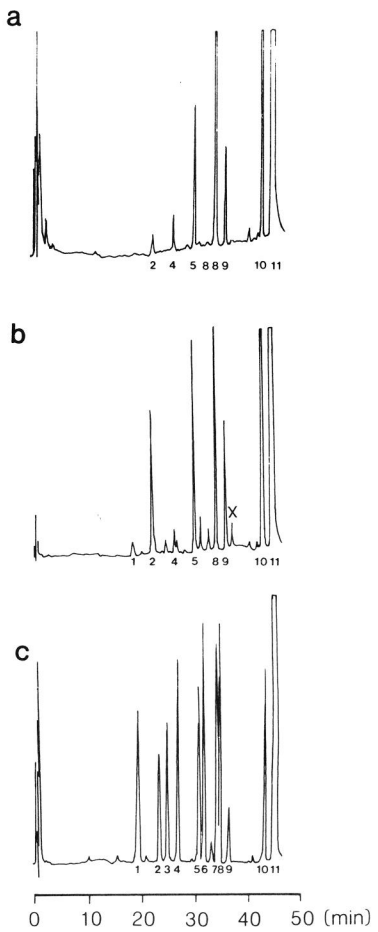

Fig. 4. Testosterone hydroxylation: representative chromatograms of (a) an extract of a microsomal incubation of 24 hr old hepatocytes, (b) an extract of a cellular incubation of a 24 hr old hepatocyte monolayer, and (c) a mixture of authentic standards (Absorbance A_{254}):1, 15β-OHT; 2, 6β-OHT; 3, 19-OHT; 4, 7α-OHT; 5, 16α-OHT; 6, 16β-OHT; 7, 12β-OHT; 8, 11β-OHT (internal standard); 9, 2α-OHT; 10, androstenedione; 11, testosterone; X, unknown metabolite with Rf=0.83. Peaks are appointed only when retention time correlated with a standard.

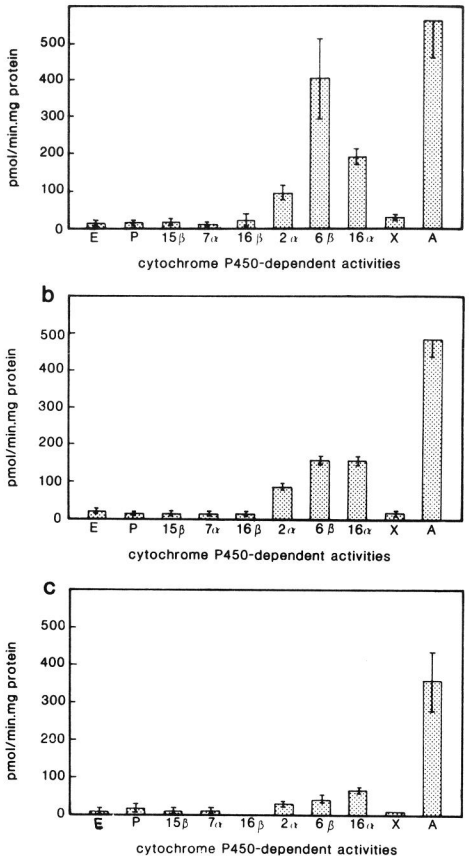

Fig. 5. Profiles of cytochrome P450-dependent activities measured directly in monolayers of cultured hepatocytes after 4 hr (a), 24 hr (b) and 96 hr culture (c). Values represent means ± SD, n = 3 rats. For abbreviations, see legend to Fig. 1.

Profile of cytochrome P450-dependent activities in hepatocyte monolayers.

Cytochrome P450-dependent activities determined in intact monolayers and expressed per mg cellular protein are shown in Fig. 5. If one compares these activities in microsomes (Fig. 1; activities expressed per mg microsomal protein) with the corresponding activities in monolayers (i.e. prepared from an equal amount of cells), the rate of product formation was 10 times higher in intact cells than in the microsomes, taking into account that in the preparation of microsomes there is a 30% loss of microsomal protein. The metabolic profile, however, appeared to be virtually the same (Fig. 5 vs Fig. 1c-d). Low activities were measured towards the substrates 7-ethoxy-resorufin and 7-pentoxyresorufin. Moreover, formation of hydroxy metabolites of testosterone in intact hepatocytes showed the same pattern towards the specific metabolites 15β-OHT, 7α-OHT, 16β-OHT and 2α-OHT as determined in microsomes of cultured hepatocytes. Both the EROD and PROD activities measured in 4 hr old primary cultures (4.3 ± 1.5 and 6.8 ± 1.9 pmol/min per mg cellular protein, respectively) were

lower than in 24 hr old cells (7.0 ± 1.9 and 18.4 ± 3.2 pmol/min per mg cellular protein, respectively). In contrast, a high degree of correlation between the decline in formation of testosterone metabolites measured in intact hepatocytes and in microsomes of cells during culture was seen for most of the metabolites of testosterone (r = > 0.938 for all metabolites except 7α-OHT (r = -0.055) and androstenedione (r = 0.788)). The rapid decline in hydroxylation at the 6β site of testosterone during the first 24 hr in culture was also seen in intact monolayers, as measured in microsomes from hepatocytes. The change in relative activity in the formation of 6β-OHT during culture is therefore not due to the preparation of microsomes. The formation of 7α-OHT and, to a lesser degree, androstenedione determined in intact hepatocytes remained at the same level during culture, which is in contrast to the decline measured in microsomes. As a result, small differences in the distribution of catalytic activities in intact hepatocytes occur during culture (Table 3).

Discussion

In untreated rats isoenzymes of P450 are hard to determine immunochemically because their levels are usually below the detection limit. Although others [11,12] could detect various isoenzymes (e.g. P450 1A1/2, P450 2A1, P450 2B1/2, P450 2C6, P450 2C11, P450 3A1) in untreated animals, we could detect only minor amounts of P450IA2, P450 2C11, P450 2E1 and P450 3A in Riv.Tox(M)-Wistar rats. This is in full agreement with the low content of cytochrome P450 of 85 pmol per mg cellular protein inRiv.Tox(M) rats whereas these levels were 225 and 500 pmol per mg cellular protein in other studies [11,12]. Besides differences in strain, the lower content of cytochrome P450 is probably also due to differences in diet. In our study, we chose to feed the rats a semi-synthetic diet which most probably has a low induction potential. Because culture medium is supposed to be free of known inducers, we expect minimal changes in cytochrome P450 isoenzyme profile to occur in culture due to differences in environmental exposure of the cells *in vivo* and *in vitro*.

In cultured hepatocytes spectrally detectable cytochrome P450 is lost more rapidly than immunochemically detectable cytochrome P450 [11,12]. Thus, levels of apoprotein may not directly reflect metabolic activity. In this study we used a broad spectrum of specific catalytic activities of different isoenzymes of cytochrome P450 to describe the profile of isoenzymes in freshly isolated hepatocytes and in liver cells in culture for several days. In agreement with others [5,32], no differences could be detected between cytochrome P450-catalysed activities measured in microsomes prepared from freshly isolated cells and whole livers. Furthermore, monitoring enzyme activities in microsomal preparations of cultured hepatocytes revealed the well-known rapid loss of cytochrome P450 (see rev. [5]), but also a decline of all cytochrome P450-dependent enzyme activities determined.

In contrast to previous suggestions that qualitative changes in the P450 isoenzyme profile with time in culture may occur favouring activity towards P450IA substrates

[5,32,33], no such changes in the profile of cytochrome P450-dependent activities could be detected in our study using e.g. 7-ethoxyresorufin as a substrate [13].

If we consider the battery of determinations as a good representation of total biotransformation activities, a comparison can be made of the distribution of different catalytic activities in the course of culture time (Table 2). During culture the distribution of most catalytic activities appeared to remain constant. However, the formation of 6β-OHT and androstenedione, expressed as a percentage of the summation of determined activities, changed during the first 24 hr in culture, but remained stable from 24 to 96 hr in culture.

It was of interest to determine whether the changes in catalytic activities as measured for the formation of 6β-OHT and androstenedione were due to selective destruction of isoenzymes during microsome preparations. Furthermore, because of the low recovery of cytochrome P450 during preparations of microsomes from hepatocytes (30%), more direct methods of determination of metabolic activities in intact hepatocytes would be desirable. Enzyme assays were developed for EROD, PROD and testosterone hydroxylation without disrupting intact monolayers. The advantages are obvious. Because no disruption of cells is needed, the endoplasmic reticulum remains intact and no possible enzyme damage due to homogenization occurs [34]. Smaller amounts of cells can be used for a single measurement because no cell material is lost during harvesting and microsome preparation. Our results show that the metabolic activities in intact cells were ca. 3 times higher than in microsomes. Furthermore, no extra cofactors are needed [18]. Using only a balanced salt solution and substrate, without extra cofactors, we consider such enzyme assays with intact hepatocytes more representative of the *"in vivo"* situation. During enzyme assays in intact cells, however, substrates and products should be able to pass the membrane and possible conjugation reactions have to be taken into consideration [4]. In our study, no conjugates of testosterone hydroxy metabolites could be detected in intact hepatocytes while testosterone itself was glucuronidated. Glucuronidation of testosterone metabolites in intact hepatocytes is apparently prevented by competition with the high amount of testosterone in the assay.

The high EROD, PROD and testosterone hydroxylation activities in primary cultured cells as compared with microsomal incubations prepared from cultured cells is remarkable. Whether this is due to enzyme damage and/or disturbance of physiological "routes" for substrates or product release, is not clear. In spite of this difference in metabolic activity, the same rapid decline of catalytic activities during culture was measured in microsomes from cultured hepatocytes as in intact cells. A few discrepancies were seen. After 24 hr in culture the EROD and PROD activities in intact cells were about twice as high as in 4 hr old cultures. Such a difference between catalytic activity in 4 and 24 hr old primary cultures was not seen in the hydroxylation of testosterone. In agreement with our study, Edwards et al. [35] reported the same low activity in ethoxycoumarin deethylation (ECOD) activity in 4 hr old primary cultures as compared to 24 hr old cultures. From 24 to 96 hr in culture, however, the EROD, PROD and ECOD [35] activities decreased at the same rate as all the other activities determined. The low activities measured after 4 hr in culture may be caused by less optimal cellular uptake of this type of substrates immediately after cell attachment because of other processes demanding energy.

The formation of 7α-OHT in intact cells remained at the same level during culture, indicating no decline of P450 2A1 [15] during culture. This is in remarkable contrast with microsomal incubations derived from cultured cells. Testosterone hydroxylation at the 6β site is catalysed by several cytochromes P450 when purified, namely P450 1A1, P450IA2, P450 2C9 and P450 3A [2,36]. In microsomes, however, no significant contributions of P450IA and P450 2C9 to 6ß-hydroxylation of testosterone could be detected [36]. Therefore, the decline of 6β-OHT during the first 24 hr in culture probably does reflects a decrease in P450 3A. The latter is in agreement with a decline of P450 3A apoprotein level during culture. A diminishing level of P450IA2 during culture was also reported by Guengerich [12].

In conclusion, metabolic activities measured in intact cultured hepatocytes are much higher than in microsomal incubations. This is only partly due to a low recovery of cytochrome P450. The unimpaired endoplasmic reticulum, the absence of possible enzyme damage due to homogenization and/or the possible more rapid release of products may contribute to this discrepancy. Thus, measurements of biotransformation activities in intact monolayers can be a convenient and reliable method to determine P450-dependent metabolic profiles.

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CHAPTER 3

COMPARISON OF CYTOCHROME P450 ISOENZYME PROFILES IN RAT LIVER AND HEPATOCYTE CULTURES

The effects of model inducers on apoproteins and biotransformation activities

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Abstract

The metabolic profile of seven subfamilies of cytochrome P450 (P450 1A, 2A, 2B, 2C, 2E, 3A, 4A) was studied in rat liver (*in vivo*) and in primary hepatocyte cultures (*in vitro*) after treatment with various inducers. The dealkylation of 7-ethoxyresorufin (EROD) and 7-pentoxyresorufin (PROD), aniline 4-hydroxylation and the regio- and stereoselective hydroxylation of testosterone were measured to characterize the isoenzyme pattern in intact hepatocytes and in liver microsomes. Occurrence of isoenzyme apoproteins was determined using Western blotting. Primary cultures of rat hepatocytes retain the capacity to respond to inducers of isoenzymes belonging to six different subfamilies (P450 1A, 2A, 2B, 2C, 3A and 4A). Treatment of cells with β-naphthoflavone revealed a P450-activity profile similar to *in vivo*, viz. a highly induced EROD (P450 1A1), a small enhancement of testosterone 7α-hydroxylation (P450 2A) and a marked reduction in 2α- and 16α-hydroxylation (P450 2C11). Exposure of cultured cells to phenobarbital resulted in a higher testosterone 16β-hydroxylation (reflecting P450 2B), though to a lesser extent than *in vivo*. The induction of P450 3A due to both phenobarbital and dexamethasone, as mirrored by 6β- and 15β-hydroxylation of testosterone, was the same in cultured hepatocytes and *in vivo*. Treatment of cells with clofibric acid resulted in an induction profile similar to the one observed in liver microsomes from clofibrate-treated rats: the apoprotein P450 4A as well as the apoprotein P450 2B1/2 and its associated activities (PROD and testosterone 16β-hydroxylation) were induced. Isoniazid, a known *in vivo* inducer of P450 2E1 and aniline 4-hydroxylation, did not change any of the determined P450-dependent activities *in vitro*.

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Introduction

Cytochrome P450 comprises a family of haemoproteins that are involved in the metabolism of a wide variety of endogenous and exogenous compounds. Some forms of this enzyme are present constitutively, whereas others are induced to high levels of expression upon exposure to certain foreign compounds [1-4]. The multigenic superfamily of cytochrome P450 has been categorized into nine different subfamilies according to resemblance between their amino acid and gene sequences [5]. Each subfamily can be characterized by its inducibility [1,2]. For example, P450 1A1 and P450 1A2 are induced by polycyclic hydrocarbons, whereas P450 2B is the major subfamily induced by phenobarbital (PB). Treatment of animals with glucocorticoids like dexamethasone (DEX) results in an induction of a third subfamily, P450 3A. In untreated male rats P450 1A1 and P450 2B are barely detectable whereas relatively high levels of 1A2, 2C6, 2C11, 3A1 and 2E have been found [1,6]. The isoenzymes differ from each other in substrate specificity and catalytic activity [2,7,8]. Therefore, changes in the isoenzyme pattern of cytochrome P450 have major implications for the metabolism and toxicity of many compounds [2].

Primary cultures of hepatocytes are widely used to study various aspects of cytochrome P450 regulation, drug metabolism, and related cytotoxicity [9-11]. Although there is a rapid decline of the basal level of cytochrome P450 during the first 24 hr in culture (for review see Ref. 11), primary cultures of rat hepatocytes retain the capacity to respond to some P450-inducers by increasing the synthesis of specific mRNAs and proteins and by enhancing associated catalytic activities [12-16]. Therefore, primary hepatocyte cultures may be used to study the capacity of various xenobiotic compounds to change the isoenzyme profile and the subsequent effects of these changes on biological processes. A more detailed comparison not only of the major induced forms of P450, but also of the total P450-dependent metabolic profile, is needed in order to establish if primary cultures represent the *in vivo* situation.

Recently, we studied the time-related decrease in culture of several P450 dependent activities and compared the activities as measured in intact monolayers and in microsomes prepared from parallel cultured cells [17]. In the present study we used five different model inducers, known to induce specifically one P450 subfamily (β-naphthoflavone, 1A; phenobarbital, 2B; isoniazid, 2E; dexamethasone, 3A; and clofibrate, 4A) to describe their influences on the metabolic profile of P450 isoenzymes. We compared the induction ratio of the dealkylation of 7-ethoxyresorufin (EROD) and pentoxyresorufin (PROD), and the regio- and stereoselective hydroxylation of testosterone as measured in intact monolayers of hepatocytes and in liver microsomes from *in vivo* treated rats. Western blotting was used to compare enzyme activities to the occurrence of P450 isoenzyme apoproteins.

Materials and methods

Materials

PB and corn oil were obtained from O.P.G. (Utrecht, The Netherlands). Clofibrate (2-(4-chlorophenoxy)-2-methylpropanoic acid ethyl ester, CLOF) was supplied by Centra Farm (Belgium). Isoniazid (INH) and clofibric acid (ethyl-2-(4-chlorophenoxy)-2-methylpropionic acid, CLOFA) were purchased from Janssen Chimica (Beerse, Belgium). Newborn calf serum was obtained from Gibco Europe (Breda, The Netherlands). Williams' E medium, β-naphthoflavone (BNF), androstenedione, DEX, 11β- and 16α-hydroxytestosterone (11β- and 16α-OHT) were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). 2α-OHT was a gift from Prof D.N. Kirk (Queen Mary College, University of London). 15β- and 12β-OHT were gifts from G.D. Searle and Co. (Skokie, IL, U.S.A.), 6β-, 7α-, 16β- and 19-OHT were obtained from Steraloids (Wilton, NH, U.S.A.). All other chemicals were of analytical grade. Monoclonal antibodies towards P450 1A1/2, P450 2B1/2 and P450 3A were kind gifts from Dr P.J. Kremers, Université de Liège, Belgium. A polyclonal antibody towards P450 2E1 was a gift from Dr I. Johansson, Karolinska Institutet, Stockholm, Sweden and a polyclonal antibody towards P450 4A was a gift from Dr G.G. Gibson, University of Surrey, Guildford, U.K. Secondary antibodies were obtained from Dakopatts a/s, Glostrup, Denmark.

Animals

Male Wistar rats (Riv.Tox(M), RIVM, Bilthoven, The Netherlands), weighing 180 - 250 g, were fed a TNO-Institute grain based open-formula diet (TNO-Toxicology and Nutrition Institute, Zeist, The Netherlands) (*ad libitum*) and had free access to drinking water. PB and INH were given as a 0.1% solution in drinking water for 5 and 10 days, respectively. BNF (40 mg/kg) was given by i.p. injection once daily for 4 days in 0.5 ml corn oil. DEX (300 mg/kg) and CLOF (250 mg/kg) were given by gastric intubation once daily for 3 days in 0.5 ml corn oil. Control rats received either corn oil i.p. for 4 days or corn oil p.o. for 3 days or no treatment at all.

Cell isolation and culture

Hepatocytes were isolated from untreated male rats (same strain and diet as in *in vivo* studies) using a two step collagenase perfusion technique [18]. The cells were plated on 6- or 9-cm tissue culture dishes (Sterilin) at a density of 4 or 8 x 10^6 cells/dish in 4 or 10 ml Williams' E medium, respectively. Media were supplemented with 3% (v/v) newborn calf serum, 1 μM insulin, 10 μM hydrocortisone and 50 mg/l gentamycin. Cells were incubated in a humidified atmosphere of air (95%) and CO_2 (5%) at 37°C. After 4 hr in culture, medium was replaced. Thereafter, media were refreshed every 24 hr.

After a total preincubation period of 24 hr, the inducers, suspended or dissolved in dimethyl sulfoxide (DMSO), were added to the culture media to give final concentrations of 1.5 mM PB, 50 μM BNF, 0.6 μM DEX, 100 μM INH or 1 mM clofibric acid (CLOFA), the latter being the active analogue of CLOF. An equal amount of DMSO was added to the control cultures (final DMSO concentration 0.1% v/v).

Preparation of microsomes

Whole livers: Livers from control and treated animals were perfused with ice-cold saline and homogenized in 0.15 M KCl containing 0.1 mM EDTA using a Potter-Elvehjem glass-Teflon homogenizer. Microsomes were prepared by centrifugation (2 x 20 min, 9000 ***g***; supernatant 60 min, 105,000 ***g***). The microsomal pellet was resuspended in sodium-phosphate buffer (0.1 M, pH 7.8) containing 0.1 mM EDTA, frozen quickly in liquid N_2 and stored at -70°C.

Cultured hepatocytes: Per treatment group seven 9-cm culture dishes were washed with ice-cold PBS and scraped with a rubber policeman in 1 ml PBS/dish. Cells were centrifuged (3 min, 50 ***g***), the supernatant was replaced by 2.5 ml 0.15 M KCl containing 0.1 mM EDTA and kept at -70°C. Microsomes were prepared as described previously [17].

Biochemical determinations

Cytochrome P450 and protein

Contents of protein and total cytochrome P450 were determined according to Rutten *et al.* [19].

Ethoxy- and pentoxyresorufin O-dealkylation (EROD and PROD)

Fluorimetric determinations of liver and hepatocyte microsomal EROD and PROD activities were performed according to the method of Burke *et al.* [7] using a Cobas-Bio centrifugal analyser, equipped with a spectrofluorimeter. Less than 200 µg microsomal protein was used in the incubation mixture of 0.1 M phosphate buffer (pH 7.4) with a final volume of 320 µl. Substrate was first dissolved in DMSO, diluted 1:10 with buffer, and added to the incubation mixture (final concentration DMSO 0.1% v/v). Substrate concentrations of 5 µM were used.

Determinations directly in intact hepatocytes were as previously described [17]. In short, hepatocyte monolayers were washed twice with Hanks' balanced salt solution (HBSS) gassed with carbogen (95% O_2 /5% CO_2, v/v). The incubation was initiated by adding 4 ml HBSS (37°C) containing 5 µM substrate and 10 µM dicumarol. Fluorescence was determined after 5 and 15 min (EROD) or 10 and 20 min (PROD) using a Kontron SFM 25 fluorimeter.

Aniline 4-hydroxylation (AH)

The AH activity in liver and hepatocyte microsomes was determined according to Chhabra *et al.* [20], using an aniline concentration of 15 mM. The reaction was stopped after 45 min at 37°C, by adding 0.5 ml 20% TCA on ice. After centrifugation (5 min, 100 ***g***), the formation of 4-aminophenol was determined spectrophotometrically at 630 nm, according to Imai *et al.* [21].

Testosterone hydroxylation

Determinations in microsomal preparations were carried out in 1 ml incubation mixture containing potassium-phosphate buffer (50 mM, pH 7.4), $MgCl_2$ (3 mM), EDTA (1 mM), $NADP^+$ (1 mM), glucose 6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (1 unit/ml), testosterone (250 µM) and 200-300 µg microsomal protein. Incubation mixture and microsomes were mixed while standing on ice. Reactions were started by heating the mixture in a 37°C water bath and stopped after 15 min by addition

of 6 ml dichloromethane. Extraction and subsequent analysis of metabolites by HPLC were performed as previously described [17]. 11β-OHT was used as internal standard.

Determinations of testosterone hydroxylation activity directly in intact monolayers were as previously described [17]. In brief, cells were washed twice with HBSS, and incubated with 4 ml HBSS containing 250 μM testosterone. After 15 min, testosterone and its metabolites were extracted with dichloromethane and subsequently analysed using HPLC.

Gel electrophoresis and immunoblotting

Separation of microsomal proteins was carried out on a Biorad mini Protean II cell applying the sodium dodecyl sulphate polyacrylamide gel electrophoresis discontinuous system of Laemmli [22], using a 4% stacking gel and a 12.5% separating gel (whole livers: 2 μg microsomal protein/lane; cultured cells: 4 μg microsomal protein/lane). The resolved proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) sheets (Millipore) at 30 V overnight according to Towbin *et al.* [23] and immunochemically stained using antibodies directed to P450 1A1/2, P450 2B1, P450 2E, P450 3A1/2 and P450 4A1. PVDF sheets were rinsed in Tris-buffered saline containing 0.3% Tween 20 (TBST) for 5 min, blocked in TBST containing 1% bovine serum albumin for 1 hr at room temperature, and thereafter incubated with primary antibody. After 1 hr, the sheets were washed 5 times in TBST and incubated with either anti-mouse or anti-rabbit IgG for 1 hr. Both IgG preparations were conjugated with alkaline phosphatase and colour was developed with a mixture of 5-bromo-,4-chloro,3-indole toluidine phosphate and *p*-nitrobluetetrazolium chloride.

Statistical analysis

Results have been expressed as means ± SD, where appropriate. Statistical analysis was performed by Students' *t*-test, for unpaired samples. The null-hypothesis was rejected at $P \leq 0.05$.

Results

In Fig. 1 the change in testosterone 15β-hydroxylation after exposure to different model inducers during culture is shown as a representative example. In this study *in vitro* induction ratios are based on the activity measured in 96-hr-old intact hepatocytes treated for 72 hr with inducer, compared to the activity in parallel cultured cells treated for 72 hr with DMSO alone.

Fig. 2 shows the elution profiles of testosterone metabolites obtained using liver microsomes from untreated rats (A,C) and intact monolayers of 96-hr-old rat hepatocytes (B,D). In untreated intact cells (Fig. 2B) relatively high levels of androstenedione (peak 9) and relatively low levels of 7α-OHT (peak 3) are formed compared to the activity in liver microsomes from control rats (Fig. 2A). We reported earlier that the testosterone metabolite profile using liver microsomes from untreated rats was similar to the metabolite profile when microsomes prepared from cultured cells were used in the incubation [17]. Treatment with model inducers did not affect the basal differences

between microsomes and intact cells towards the formation of 7α-OHT and androstenedione. As an example, the testosterone metabolite profile after exposure to clofibrate *in vivo* and clofibric acid *in vitro* are shown in Fig. 2C and D. These resultsindicate that induction ratios can be compared only as calculated from the activity in treated rats and hepatocytes, divided by the corresponding activities in their matching controls.

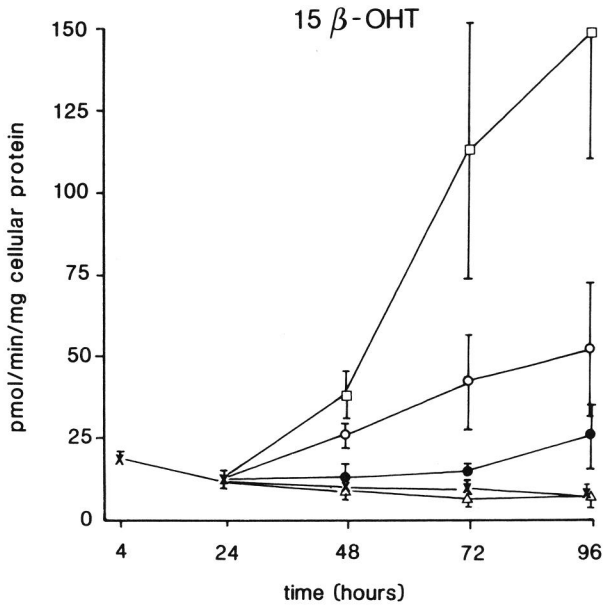

Fig. 1. Change in 15β-hydroxylation of testosterone measured in intact hepatocyte monolayers after exposure to vehicle (DMSO) only (x), β-naphthoflavone (△), phenobarbital (○), clofibric acid (●) and dexamethasone (□). After a preincubation of 24 hr, hepatocytes were exposed to the inducing compound for another 72 hr. Data are means ± SD from three different experiments.

In Fig. 3 an overview is given of the induction ratios of several biotransformation activities *in vivo* and *in vitro*. Table 1 shows semi-quantitative determinations of apoprotein levels of several P450s in microsomes prepared from cultured hepatocytes and whole liver. The original data concerning the P450-dependent activities, including the statistical test results, are given in Table 2 (*in vivo*) and Table 3 (*in vitro*).

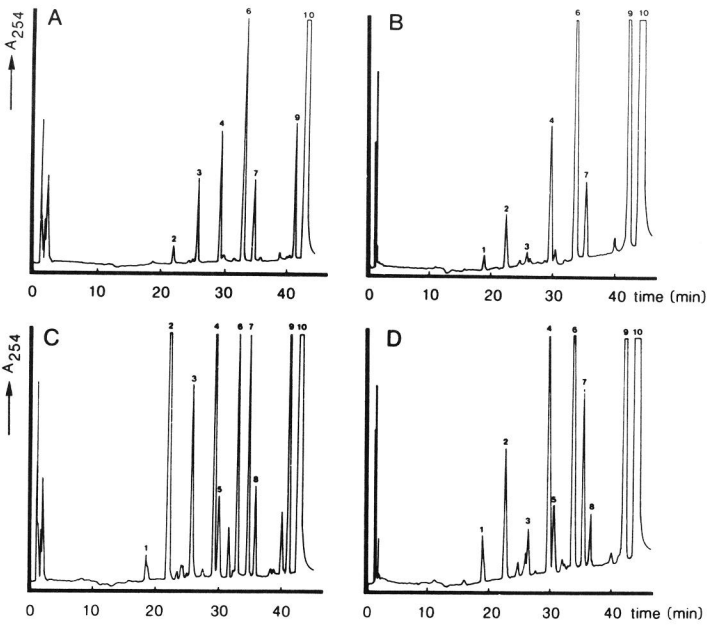

Fig. 2. Elution profiles of testosterone metabolites determined in microsomes from rat liver and in intact monolayers of cells. A: *in vivo* control; B, control 96-hr-old hepatocytes; C, *in vivo* rats treated with clofibrate for three days; D, 96-hr-old hepatocytes treated with clofibric acid for 72 hr. Peak identification: 1, 15β-OHT; 2, 6β-OHT; 3, 7α-OHT; 4, 16α-OHT; 5, 16β-OHT; 6, 11β-OHT (internal standard); 7, 2α-OHT; 8, X, unknown metabolite; 9, androstenedione; 10, testosterone.

Exposure to β-naphthoflavone

Treatment of rats with BNF *in vivo* resulted in the well-known induction of EROD activity (24-fold; Fig. 3a), related to an enhanced level of P450 1A apoproteins (Table 1). BNF increased the 7α-hydroxylation of testosterone 3.5-fold, but decreased testosterone 2α- and 16α-hydroxylation in the liver. The PROD activity in microsomes from BNF-treated rats was enhanced (3-fold), although no P450 2B1/2 apoprotein levels could be detected (Table 1). Exposure of hepatocyte cultures to BNF resulted in a similarmetabolic profile to the one observed after exposure to BNF *in vivo* (EROD 24- and 27-fold enhanced, *in vivo* and *in vitro*, respectively), though no enhancement of PROD activity was measured in intact monolayers after BNF treatment. In contrast, in microsomes prepared from BNF-treated cells a similar enhancement of PROD activity was measured as *in vivo* microsomes (results not shown). The characteristic decrease in 2α- and 16α-hydroxylation of testosterone after BNF treatment was also seen *in vitro*. Immunochemical analysis showed the presence of both P450 1A1 and 1A2, although the level of 1A2 is lower in culture than *in vivo* (Table 1).

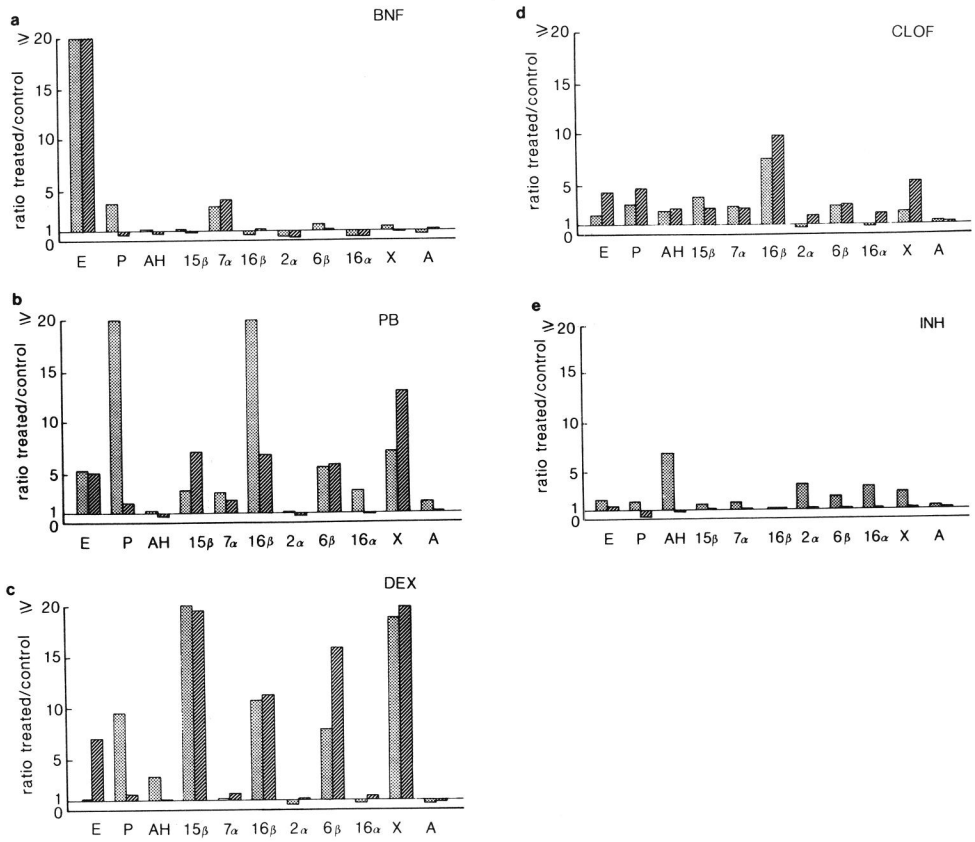

Fig. 3. Induction ratios (treated/matching control) of cytochrome P450-dependent activities measured in liver microsomes from rats treated with different inducers *in vivo* and in intact cultured hepatocytes after exposure to the same inducers. a, β-naphthoflavone (BNF); b, phenobarbital (PB); c, dexamethasone (DEX); d, clofibrate (CLOF(A)); e, isoniazid (INH). ▒, *in vivo*; ▨, *in vitro*. ≥ 20 indicates an induction ratio of 20 or higher. For duration of treatment see Materials and Methods. Because of a 16β-OHT activity below the detection limit in 96-hr-old control cultures, minimal ratios for this activity *in vitro* are given. Legends: E, EROD; P, PROD; AH, aniline 4-hydroxylation, 15ß, 7α, 16ß, 2α, 6ß, 16α, all hydroxymetabolites of testosterone; X, unknown metabolite of testosterone; A, androstenedione.

Table 1. Immunochemical detection of apoprotein levels of different P450 isoenzymes, *in vivo* and *in vitro* after treatment with phenobarbital (PB), ß-naphthoflavone (BNF), dexamethasone (DEX), clofibrate/clofibric acid (CLOF(A)) and isoniazid (INH).

	control	BNF	PB	DEX	CLOF	INH
In vivo treatment						
P450 1A1	-	+++	-	-	-	-
P450 1A2	+	++	-	-	-	+
P450 2B1/2	-	-	+++	++	++	-
P450 2E	+	+	+	+	++	+++
P450 3A	+	-	+	+++	+	-
P450 4A	-	-	-	-	+++	-
In vitro treatment						
P450 1A1	-	+++	-	+	+	-
P450 1A2	±	+	-	-	-	-
P450 2B1/2	-	-	±	±	+	-
P450 2E	+	+	+	+	+	+
P450 3A	±	-	±	+++	±	-
P450 4A	-	-	-	-	+++	-

In vivo, 2 µg microsomal protein per lane; *in vitro*, 4 µg microsomal protein per lane; Key: - , isoenzyme not detected; ±, isoenzyme weakly detected; +, isoenzyme easily detected; ++, isoenzyme strongly detected; +++, isoenzyme very strongly detected.

Exposure to phenobarbital

Treatment of rats with PB caused a large induction of cytochrome P450 2B *in vivo* (Table 1) and resulted in an associated increase in liver microsomal PROD activity and 16β-hydroxylation of testosterone (67- and 36-fold, respectively; Fig. 3b). EROD activity was enhanced 5-fold, whereas no P450 1A could be detected immunochemically in PB-microsomes (Table 1). The production of 15β-, 7α-, 6β- and 16α-OHT and an unknown metabolite with a relative retention time towards testosterone of 0.83 (probably 18-OHT [21], to be called X in this text) were all increased after PB treatment. In contrast to PB treatment *in vivo*, exposure of hepatocytes to PB revealed only minor levels of P450 2B1/2 proteins, which was mirrored in low PROD, 16β- and 16α-testosterone hydroxylation activities (Fig. 3b). On the other hand PB induced P450 3A apoprotein levels both in cell culture and *in vivo* (Table 1), associated with enhanced production of 6β-OHT, 15β-OHT and compound X, the latter two even more enhanced *in vitro* compared to the induction *in vivo*. The enhancement of EROD activity in PB-treated hepatocytes was to a similar extent as *in vivo*.

Table 2. Profile of P450-dependent activities in liver from rats treated with different inducers.

	control	co-ip	co-po	BNF	PB	DEX	CLOF	INH
P450	372 ± 165	350 ± 23	290 ± 36	879 ± 138*	661 ± 151*	1211 ± 120*	782 ± 54*	417 ± 23
EROD	44 ± 18	110 ± 21*	53 ± 11	2601 ± 389*	241 ± 24*	106 ± 23*	113 ± 37*	98 ± 46*
PROD	5 ± 3	11 ± 2*	8 ± 1	43 ± 9*	322 ± 109*	76 ± 5*	24 ± 3*	9 ± 3*
AH testosterone	446 ± 194	633 ± 206	424 ± 83	761 ± 97	580 ± 250	1477 ± 472*	1055 ± 94*	3206 ± 790*
15ß	9 ± 4	14 ± 7	9 ± 4	14 ± 2	32 ± 7*	541 ± 72*	34 ± 14*	15 ± 7
7α	76 ± 51	143 ± 77	107 ± 53	506 ± 140*	237 ± 93*	125 ± 47	323 ± 95*	141 ± 50
16ß	18 ± 6	16 ± 9	10 ± 2	10 ± 3	671 ± 135*	116 ± 26*	81 ± 13*	20 ± 6
2α	254 ± 57	964 ± 617	551 ± 55*	393 ± 171	287 ± 39	313 ± 66*	424 ± 112	963 ± 239*
6ß	223 ± 147	537 ± 85	449 ± 146	923 ± 151*	1272 ± 107*	4136 ± 961*	1462 ± 701	558 ± 177*
16α	460 ± 108	1554 ± 1025	927 ± 110*	649 ± 278	1541 ± 280*	708 ± 84*	794 ± 251	1598 ± 385*
X	19 ± 8	48 ± 2	44 ± 10	68 ± 5*	139 ± 21*	871 ± 137*	105 ± 44*	55 ± 12*
Andr	529 ± 229	692 ± 252	508 ± 60	481 ± 67	1142 ± 265*	349 ± 38*	674 ± 106*	760 ± 110

The values are expressed as pmol/min/mg microsomal protein and are means ± SD (n=3 rats, except control n=5 rats). * $p \leq 0.05$ when compared to matching control; Inducers phenobarbital (PB) and isoniazid (INH) compared to control, β-naphthoflavone (BNF) compared to control-ip (co-ip), clofibrate (CLOF) and dexamethasone (DEX) compared to control-po (co-po).

Table 3. Profile of P450-dependent activities in primary rat hepatocyte cultures after treatment with various inducers for 3 days.

	control 24 hr	control 96 hr	BNF	PB	DEX	CLOFA	INH
P450[$]	170 ± 53	136 ± 65	174 ± 3	145 ± 24	170 ± 115	131 ± 116	122 ± 55
EROD	7 ± 2	4 ± 2	104 ± 8*	20 ± 3*	29 ± 4*	18 ± 3*	6 ± 2
PROD	18 ± 3	5 ± 2	3 ± 1	11 ± 3*	9 ± 2*	24 ± 4*	2 ± 1*
AH[$]	238 ± 97	64 ± 30	31 ± 2	38 ± 5	70 ± 5	115 ± 6	91 ± 14
testosterone							
15β	11 ± 1	7 ± 2	8 ± 2	54 ± 20*	150 ± 40*	25 ± 11	10 ± 2
7α	13 ± 5	10 ± 5	41 ± 10	23 ± 8*	17 ± 5	16 ± 7	11 ± 4
16β	5 ± 2	< 2	< 2	14 ± 1	22 ± 2	22 ± 1	< 2
2α	80 ± 7	30 ± 6	9 ± 3*	20 ± 5*	35 ± 7	59 ± 7*	31 ± 10
6β	160 ± 12	41 ± 17	42 ± 11	245 ± 99*	682 ± 221*	126 ± 63	45 ± 14
16α	155 ± 11	65 ± 6	29 ± 5*	56 ± 12	96 ± 18*	143 ± 7*	66 ± 7
X	15 ± 1	5 ± 4	4 ± 1	71 ± 31*	236 ± 68*	27 ± 17*	4 ± 1
Andr	493 ± 49	360 ± 87	447 ± 104	393 ± 112	305 ± 90	444 ± 38	397 ± 86

The values are expressed as pmol/min/mg cellular protein and are means ± SD (n=3 rats).* $p \leq 0.05$ when compared to 96-hr-old control hepatocytes; Inducers: BNF, β-naphthoflavone; PB, phenobarbital; DEX, dexamethasone; CLOFA, clofibric acid; INH, isoniazid. After a preincubation period of 24 hr, hepatocytes were exposed to the inducing compound for another 72 hr. [$] P450 content and aniline 4-hydroxylation (AH) were determined in microsomes prepared from cultured hepatocytes and are both expressed per mg microsomal protein. P450 content in freshly isolated cells was 343 ± 84 pmol/mg microsomal protein.

Exposure to dexamethasone

DEX, an inducer of subfamily P450 3A (Table 1), increased the production of 15β-OHT (63-fold) and compound X (20-fold) *in vivo* (Fig. 3c). The PROD activity as well as the hydroxylation activity towards the 16β- and the 6β-site of testosterone were highly induced, 10-, 11- and 8-fold, respectively. Immunochemical studies of DEX-microsomes revealed an induction of both P450 3A and P450 2B apoprotein levels *in vivo* (Table 1). Treatment of cultured hepatocytes with DEX resulted in a similar induction profile concerning the testosterone metabolites, although the induction ratios of testosterone 6β-hydroxylation and the formation of compound X (50-fold) were higher *in vitro* compared to the activity in liver after *in vivo* exposure to DEX (Fig. 3c). Unlike PB, in DEX-treated hepatocytes the production of 16β-OHT was induced to the same extent compared to the induction of this activity *in vivo*. However, the PROD activity was not enhanced *in vitro*. A remarkable difference *in vivo* was found concerning the EROD activity after DEX exposure, since in DEX-treated cells (measured in intact cells as well as in microsomes prepared from these cells) this activity was induced 7-fold, which was consistent with a small but distinct induction of P450 1A1 apoprotein *in vitro* (Table 1).

Exposure to clofibrate and clofibric acid

Treatment of rats with CLOF resulted in an induction of the apoprotein level of P450 4A, which was not found with any of the other model inducers (Table 1). Moreover, an 8-fold induction of 16β-hydroxylation of testosterone was measured (Fig. 3d), which was associated with a substantial increase in the level of apoprotein P450 2B1 (Table 1). A more modest increase (3- to 4-fold) was determined for PROD, 15β-, 7α- and 6β-OHT activities. Treatment of primary cultures with clofibric acid resulted in a metabolic profile almost similar to the one obtained after exposure *in vivo* (Fig. 3d), and in the same characteristic induction of P450 4A apoprotein (Table 1). An interesting point was the relatively high induction of P450 2B1/2 due to clofibric acid in hepatocyte culture as compared to the induction of this isoenzyme(s) in PB-treated cells (Fig. 4). In agreement, both PROD and testosterone 16β-hydroxylation were enhanced *in vitro* to a similar level as in rat liver after treatment with CLOF. In comparison to DEX exposure, treatment of cells with CLOFA revealed also a small induction of P450 1A1 apoprotein levels (Table 1) and higher EROD activity.

Exposure to isoniazid

Isoniazid increased the rate of aniline 4-hydroxylation (7-fold) *in vivo* (Fig. 3e). This activity is known to be preferentially catalysed by P450 2E and P450 1A2 [2,22]. Towards testosterone a 2- to 4-fold increase was measured in the production of 2α-OHT, 16α-OHT, 6ß-OHT and the unknown metabolite X. Both EROD and PROD activities were significantly enhanced (2-fold) as well. However, in isoniazid-treated cells only the EROD activity was increased whereas even a decrease in PROD activity was measured. No enhancement of aniline 4-hydroxylation (Fig. 3e) or P450 2E apoprotein levels (Table 1) could be detected *in vitro*.

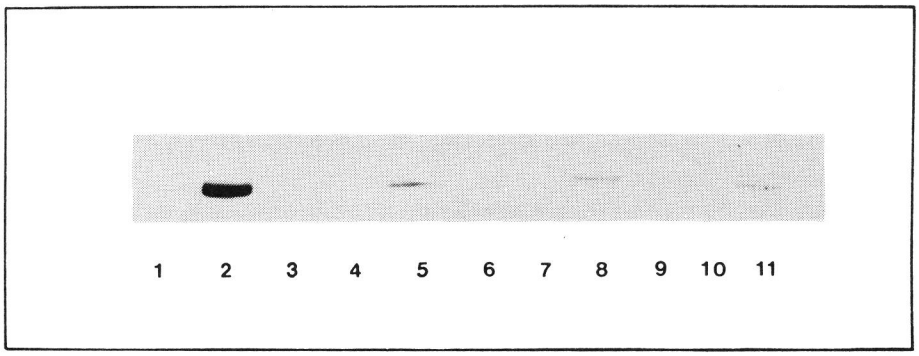

Fig. 4. Immunoblot of microsomes prepared from rat liver and cultured hepatocytes, immunochemically stained with anti-P450 2B1/2. Lanes 1-5, liver microsomes from rats treated *in vivo* (2 µg protein/lane): 1, control; 2, phenobarbital; 3, β-naphthoflavone; 4, isoniazid; 5, clofibrate. Lanes 6-11, microsomes from cultured hepatocytes (4 µg protein/lane): 6 and 9, 24-hr-old hepatocytes; 7 and 10, 96-hr-old hepatocytes treated with DMSO; 8, 96-hr-old hepatocytes treated with clofibric acid; 11, 96-hr-old hepatocytes treated with phenobarbital.

Discussion

So far, most studies of the effects of inducers on cytochrome P450 *in vitro* have concentrated on the enhancement of one or few specific P450 isoenzymes [13-16]. A direct comparison to study whether the *in vitro* changes of a broad spectrum of P450 activities, due to exposure to various model inducers, mimics the *in vivo* situation, has not been described yet. We used different specific catalytic activities supported with immunochemical detection of P450 apoproteins, to study the change in metabolic profile.

Treatment of rats *in vivo* with BNF, PB, DEX, CLOF and INH resulted in changes in P450 apoprotein pattern and associated catalytic activities which are consistent with data reported by others [2,24-26]. Treatment of hepatocytes with the same model inducers resulted to a large extent in similar metabolic profiles and induction ratios. However some marked differences can be observed.

Exposure of cultured hepatocytes to BNF resulted in a catalytic profile very similar to the *in vivo* profile: a high EROD activity (which is preferentially catalysed by P450 1A1 [7]), a moderate increase of 7α-hydroxylation (which represents P450 2A [26]) and a decrease in testosterone 2α- and 16α-hydroxylation. This decrease to *ca.* 40% of the activity measured in controls (*in vivo* as well as *in vitro*) could only be detected after treatment with BNF. P450 2C11 is known to catalyse testosterone hydroxylation at the 2α-site and to a lesser extent at the 16α-site [4]. A decrease of P450 2C11 is also observed in rats after exposure to other P450 1A inducers [1]. These results indicate a

comparable degree of down regulation of P450 2C11 *in vivo* and *in vitro* after BNF treatment.

PB is known to increase the *de novo* synthesis of apocytochrome P450 2B1/2 and P450 3A in rat liver [2,27]. P450 2B1/2 catalyses the conversion of testosterone mainly to 16β-OHT, and to a lesser extent to 16α-OHT and androstenedione [8]. However, in cultured hepatocytes P450 2B1/2 apoprotein levels and associated activities after PB treatment is low compared to the *in vivo* situation. For many years, several investigators [11,14,27,28] have been concentrating on identifying culture conditions which would permit P450 2B1/2 to be induced in cultured hepatocytes. Recently, Schuetz *et al.* [29] reported a higher enhancement of P450 2B1/2 mRNA and apoprotein level after PB treatment when hepatocytes were cultured on a reconstituted basement membrane gel. However, no associated activities i.e. formation of 16β-OHT or PROD, were reported. In a preliminary study we could detect a small induction of P450 2B1/2 mRNA levels in PB- and CLOFA-treated cells (8- and 32-fold, respectively; results not shown). Therefore, in addition to the small induction of the apoprotein P450 2B1/2 and an enhancement of the associated enzyme activities (16β-hydroxylation of testosterone and PROD), we conclude that cultured hepatocytes do respond to P450 2B inducers, especially CLOFA, with an enhanced level of mRNA, apoprotein and associated enzyme activity, although in the case of PB to a much lower level than *in vivo*. A recent study of Waxman *et al.* [30] describes a comparable *in vivo* level of P450 2B and associated enzymatic activities in PB-treated hepatocytes when cells were cultured for 10 or more days.

In contrast to the low P450 2B1/2 levels in PB-treated cells, the P450 3A apoprotein level and its associated [4] activities towards the 6β- and 15β- site of testosterone, were induced. P450 3A is also known to catalyse the formation of 18-hydroxytestosterone [31,32]. Based on the enhanced production of compound X after PB and DEX treatment and a comparable relative retention time in a similar HPLC elution profile [31], we deduce that compound X most probably is identical to 18-hydroxytestosterone.

Exposure of cultured hepatocytes to CLOFA resulted in the characteristic enhancement of the P450 4A apoprotein level as observed *in vivo* [3]. Induction of this isoenzyme *in vitro* was also reported by Lake *et al.* [16], who, in addition, determined the corresponding higher activity in 12-hydroxylation of lauric acid. No lauric acid hydroxylation was measured in this study. Beside induction of P450 4A, exposure to CLOF(A) induced almost every P450-dependent activity measured in our study *in vivo* as well as *in vitro*. These results are in contrast with data of Sharma *et al.* [3], who measured a reduced dealkylation of both ethoxyresorufin and benzphetamine in rat liver after treatment with CLOF. However, other investigators [12,16,24] reported the enhancement of several P450 isoenzymes after CLOF treatment which is consistent with our data. The reason for this discrepancy is not clear.

P450 2E is different from many other forms of P450 since it is induced by numerous structurally different compounds [32,33], many of which are known to effectively bind to the enzyme. Eliasson *et al.* [34] reported a maintenance of P450 2E in primary cultures of rat hepatocytes, provided that enzyme ligands (preventing enzyme degradation) are present in the medium. The induction of this isoenzyme *in vivo* is correlated with ligand binding and stabilization of mRNA levels, resulting in higher

protein levels [35]. However, in agreement with our study, no intion of this isoenzyme *in vitro* has been reported yet. Whether the lack of high induction of P450 2B and P450 2E *in vitro* has to be attributed to hormonal disbalance has been the subject of many research [4,27,36,37].

In this study we monitored specific biotransformation activities to study the P450 isoenzyme profile. In this regard the O-dealkylation of 7-ethoxyresorufin is often used as a reflection of the activity of P450 1A1 [7]. However, in control and PB-treated rats the EROD activity (in absence of P450 1A1) is mainly catalysed by two constitutive enzymes, P450 2C11 and P450 2C6 [38]. For this reason, we would emphasize the need of both immunochemical and catalytic activity determinations to study the P450 isoenzyme profile. The 4-fold higher PROD activity in liver microsomes from BNF-treated rats *in vivo* (in absence of P450 2B), for example, is probably the result of a catalytic activity of P450 1A1 towards this substrate. This is supported by the study of Nakajima *et al.* [38] who could inhibit PROD activity in liver microsomes from rats treated with 3-methylcholanthrene for 50% with a monoclonal antibody directed towards P450 1A. In contrast, in intact BNF-treated cells no enhancement of PROD activity was measured, whereas in microsomes prepared from similar BNF-treated cells the PROD activity was enhanced to the same level as *in vivo*. Such discrepancies between the activity in intact cells compared to the same activity in microsomes (prepared from cells) were also found for the formation of 7α-hydroxylation and androstenedione. These results may indicate that changes in substrate accessibility and/or changes in the efficiency for the electron transfer from NADPH P450-reductase to certain P450 isoenzymes are introduced during preparation of microsomes.

In conclusion, in primary rat hepatocytes several isoenzymes of cytochrome P450, namely P450 1A1, 1A2, 2A, 2C, 3A and 4A, can be changed with the same inducing compounds and to the same extent as *in vivo*. The induction of P450 2B1/2 in rat hepatocytes, however, occurs to a much lower level compared to *in vivo*. It should be noted that two distinct ways of regulation could be possible for this isoenzyme because of the different level of induction after PB and CLOF treatment *in vitro*. No induction of P450 2E could be detected in primary hepatocytes. In spite of this incompleteness for some forms of P450 we conclude that primary cultures of hepatocytes can be a useful alternative in studying the changes in biotransformation activities catalysed by the P450 1A, 2A, 2C, 3A and 4A subfamilies as well as the effects on cytochrome P450-mediated cytotoxicity.

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CHAPTER 4

THE EFFECTS OF COOKED BRUSSELS SPROUTS ON CYTOCHROME P450 PROFILE AND PHASE II ENZYMES IN LIVER AND SMALL INTESTINAL MUCOSA OF THE RAT

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Abstract

Male Wistar rats were given semi-synthetic diets supplemented with 0, 2.5, 5 and 20% cooked Brussels sprouts for 2, 7, 14 or 28 days. The effects on several cytochromes P450 and phase II enzymes (glutathione S-transferase (GST), glucuronyl transferases 1 and 2 (GT1 and GT2) and DT-diaphorase (DTD)) in liver and small intestinal mucosa were investigated. From two days of exposure onwards Brussels sprouts induced the P450 1A2 and - to a lesser extent - P450 1A1 apoprotein levels in the liver, whereas in the small intestine markedly enhanced P450 2B apoprotein levels could be detected. No enhanced P450 3A apoprotein levels were observed. The 5% and 20% sprouts diets increased the intestinal pentoxyresorufin depentylation (PROD, 4.5- to 9-fold) and the hydroxylation of testosterone at the 16α- and 16β-site (2.6- to 4.2-fold) after two days of exposure. In addition, the 20% sprouts diet also enhanced the intestinal ethoxyresorufin deethylation (EROD) activity (*ca* 5-fold), the hepatic EROD and PROD activities (*ca* 2-fold) and the formation of 6β-hydroxytestosterone (*ca* 1.6-fold); the formation of 2α-hydroxytestosterone in the liver was decreased (to *ca* 70% of the control value).

GST activity was induced both in liver (5 and 20% diet) and intestine (20% diet only) throughout the experiment. The 20% sprouts diet enhanced the hepatic DTD and GT1 activities, whereas the GT2 activity was decreased. The induction of DTD in the small intestine after two days (2.5- to 3.2-fold with 5% and 20% sprouts diet, respectively) diminished during the experiment. These results indicate that dietary exposure to cooked Brussels sprouts for a period as short as two days can affect several phase II enzymes and cytochromes P450. P450 2B and P450 1A2 are the predominant forms to be induced in the small intestine and in the liver, respectively.

Fd Chem Toxic (in press)

Introduction

Cruciferous vegetables (especially *Brassica* species e.g. broccoli, Brussels sprouts and cauliflower) have been shown to enhance the activity of biotransformation enzymes in both animals and man [for reviews see 1,2]. The toxicity of many xenobiotics depends *i.a.* on the balance between the rate of formation of reactive metabolites by Phase I enzymes (cytochromes P450, P450) and the rate of their inactivation through conjugation by Phase II enzymes. The alteration in metabolic activity by *Cruciferae* may be responsible for the associated decrease in risk of cancer described in rodent and man [3].

So far, many researches have been focused on changes in ethoxyresorufin O-deethylation, aryl hydrocarbon hydroxylation and/or ethoxycoumarin O-deethylation activities [4-6], all - more or less specifically - representing one subfamily of the P450 forms, i.e. P450 1A (nomenclature according to Nebert *et al.* [7]). Little account has been taken of the fact that other changes in the cytochrome P450 profile may also have a considerable effect on the metabolism of various xenobiotics [8]. Measuring specific activities, i.e. 7-ethoxyresorufin and 7-pentoxyresorufin dealkylation (EROD and PROD) and the regioselective hydroxylation of testosterone, together with immunochemical staining of separate P450 apoproteins, we studied the effects of cooked Brussels sprouts on the cytochrome P450 profile in liver and small intestine.

Besides the induction of mixed function oxidase activities, the enhancement of several phase II enzymes such as glutathione S-transferase (GST; EC 2.5.18), UDP-glucuronyl transferase (GT; EC 2.4.1.17) and DT-diaphorase (DTD; NAD(P)H-quinone reductase; EC 1.6.99.2) due to dietary exposure to cruciferous vegetables has been described [4,9,10]. The enhancement of these mainly detoxifying enzyme systems are associated with a decrease in tumorigenesis in a way as reported for e.g. the antioxidants butylated hydroxyanisole and butylated hydroxytoluene [11].

Thus far, in most studies rats were fed a relatively high dose of raw (freeze dried) cabbage during a relatively long period. In the human diet the majority of the cruciferous vegetables are cooked before consuming. Cooking reduces the levels of glucosinolates to *ca.* 40 % [12], the compounds in *Cruciferae* of which the decomposition products are held responsible for the changes in biotransformation activities [1,10]. In preliminary experiments, however, no decrease in GST and DTD induction ratio could be measured in rats treated with 15% cooked Brussels sprouts (26 days) compared to a diet of 15% raw Brussels sprouts (de Groot, unpublished data). Moreover, in the same study the EROD activity was even enhanced after dietary exposure to cooked sprouts. These results indicate that cooking of cruciferous vegetables may introduce other or perhaps more effective glucosinolate derivatives. It is already well established that specific break-down products of glucosinolates such as indoles, isothiocyanates and goitrins have enzyme-inducing effects [1,10].

The aim of the present study was to investigate the effects of cooked Brussels sprouts on cytochrome P450 profile and several phase II enzymes in rat liver and small intestine, at a level and exposure time more related to the situation for man. For reason of comparison with other studies, both a high level of Brussels sprouts (20%) and long exposure times (14 and 28 days) were incorporated in the experiment.

Materials and methods

Chemicals

Testosterone, 11β- and 16α-hydroxy-testosterone (11β- and 16α-OHT) were obtained from Sigma Chemical Co., St Louis, MO, USA. 2α-OHT was a kind gift from Prof D.N. Kirk (Queen Mary College, University of London), 15β-OHT was a gift from G.D. Searle and Co., Skokie, IL, USA. 6β-, 7α- and 16β-OHT were obtained from Steraloids, Wilton, NH, USA. All other chemicals were of analytical grade. Monoclonal antibodies directed towards P450 1A1/2, P450 2B1/2 and P450 3A were all kind gifts from Dr P.J. Kremers, Université de Liège, Belgium. Secondary antibody (alkaline-phosphatase labeled) was obtained from Dakopatts a/s, Glostrup, Denmark.

Animals and diet

Male Wistar rats (Crl(WI)Br), aged 6 wk, were obtained from Charles River WIGA (Sulzfeld, FRG). Animals were housed in groups of six in stainless steel cages with wire-screen bottom and front. The room temperature was maintained at 21 ± 2°C and the relative humidity at 40-70%. A light/dark cycle of 12 hr was maintained and the ventilation rate was *ca* ten air changes/hr. Food and water were available *ad libitum*. Before the experiment all rats were fed a TNO open-formula basal diet for 7 days. Rats were allocated to four diet groups of 24 animals using a computer-generated random-number table. At the start of the experiment the mean body weight was *ca* 117 g.

Brussels sprouts (stored under controlled atmospheric conditions, -1°C) were purchased from the grower (Groeneboom, Ridderkerk, The Netherlands) and kept at -20°C afterwards. At intervals of three days Brussels sprouts were defrosted and cooked for 20 min in unsalted water. Due to cooking total glucosinolate levels reduced from *ca* 7.3 to *ca* 4.9 mmol/kg (ww) sprouts. Glucosinolates were determined according to the method described by Spinks *et al.* [13]. Cooked Brussels sprouts were incorporated into a semi-synthetic diet at levels providing 2.5, 5 and 20% of the diet's dry matter. The compositions of the test diets are given in Table 1. The diets were provided as a paste and a comparable consistency of the diets was obtained by supplementation of various amounts of water. The contents of crude protein and crude fibre were equalized by compensating with casein and cellulose. Diets were kept refrigerated at 3°C until new diets were prepared (every three days). Diet and water were provided *ad libitum*. The body weights of the individual rats were recorded weekly. Food intake was measured daily. After 2, 7, 14, and 28 days rats (six animals per group) were killed by bleeding from the aorta abdominalis under ether anaesthesia. The liver and the proximal 20 cm of the small intestine were collected.

Preparation of microsomes and cytosols

All procedures were performed at temperatures ranging from 0-4°C. Microsomes and cytosol of liver were prepared according to Rutten *et al.* [14]. Livers were homogenized in 3 volumes ice-cold Tris-buffered 1.15% KCl (0.01M Tris-HCl, pH 7.4) using a Potter-Elvehjem glass-teflon homogenizer. After centrifugation for 15 min at

9000 ***g***, the post-mitochondrial fraction was centrifuged for 60 min at 105.000 ***g***. The microsomal pellet was resuspended in Tris buffered 1.15% KCl with an ultra-turrax.

Table 1. Composition of the diets.

Ingredients	Concentration (g/kg air-dry diet) Brussels sprouts			
	Control	2.5%	5%	20%
Casein	200	192	183	132
DL-Methionine	2	2	2	2
Cellulose	50	46	42	24
Mineral mix*	40	40	40	40
Vitamin ADEK prep.**	4	4	4	4
Vitamin B mixture***	2	2	2	2
Choline chloride (50%)	4	4	4	4
Corn-oil	50	50	50	50
Wheat starch (native)	498	504	511	542
Wheat starch (pregelat.)	150	131	112	-
Brussels sprouts (cooked)#	-	205	410	1640
Water added	1000	900	800	200

* The mineral mixture contained (per gram mix): 399 mg KH_2PO_4, 389 mg $CaCO_3$, 142 mg NaCl, 58 mg $MgSO_4$, 5.7 mg $FeSO_4.7H_2O$, 0.9 mg $ZnCl_2$, 0.8 mg $CuSO_4.5H_2O$, 4.6 mg $MnSO_4.2H_2O$, 0.02 mg $CoCl_2.6H_2O$, 0.08 mg $KCr(SO_4)_2.12H_2O$.

** The ADEK vitamin preparation contained (per gram): 939 mg vitamin AD concentrate, containing 2250 IU/g vitamin A and 750 IU/g vitamin D, 15 mg vitamin E, 1.0 mg vitamin K_3, 45 mg wheat starch.

*** The vitamin B mixture contained (per gram): 2.5 mg thiamine-HCl, 3.0 mg riboflavin, 10 mg pyridoxin-HCl, 12.5 mg niacin, 7.5 mg Ca-panthothenate, 0.075 mg biotin, 0.5 mg folic acid, 0.035 mg vitamin B_{12}, 964 mg ground sucrose.

\# The water content of cooked Brussels sprouts was *ca.* 88%.

Cytosol and microsomal fractions (in portions of 1 ml) were quickly frozen in liquid N_2 and stored at -80°C until use. Microsomes and cytosol of the small intestine were prepared according to Borm *et al.* [15] with modifications as described by Bogaards *et al.* [10]. Briefly, the proximal 20 cm of the small intestine was perfused with ice-cold PBS and subsequently slipped inside out over 3-mm diameter stainless steel pins mounted on a Vibro-mixer. The epithelial cells were released by vibration in PBS containing 5 mM EDTA for 40 min. The cells were repeatedly washed, suspended in 2 ml PBS and sonicated for 10 sec (amplitude 6 μm). After centrifugation at 2500 ***g*** for 20 min, the post-mitochondrial fraction was centrifuged at 105.000 ***g*** for 60 min. The microsomal pellet was resuspended in 1 ml PBS with an ultra-turrax. Microsomes and cytosol were quickly frozen in portions of 0.5 ml in liquid N_2 and stored at -80°C.

Enzyme assays

Cytochrome P450 and protein

Contents of protein and total hepatic cytochrome P450 were determined according to Rutten *et al.* [14].

Ethoxy- and pentoxyresorufin O-dealkylation (EROD and PROD)

Fluorimetric determinations of liver and intestinal microsomal 7-ethoxyresorufin O-deethylation (EROD) and 7-pentoxyresorufin O-dependentylation (PROD) activities were performed according to the method of Burke *et al.* [16] using a Cobas-Bio centrifugal analyser, equipped with a spectrofluorimeter. Less than 200 µg microsomal protein was used in an incubation mixture of 0.1 M phosphate buffer (pH 7.4) and NADPH-regenerating system with a final volume of 320 µl. Substrate was added in diluted dimethyl sulfoxide (final DMSO concentration 0.5% v/v). Substrate concentrations of 5 µM were used.

Testosterone hydroxylation

Hepatic and intestinal hydroxylation of testosterone was determined as described previously [17]. In short, microsomes (150-400 µg) were incubated in 1 ml potassium-phosphate buffer (50 mM, pH 7.4) containing 3 mM $MgCl_2$, 1 mM EDTA, 1 mM $NADP^+$, 5 mM glucose-6-phosphate, 1 unit/ml glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and 250 µM testosterone at 37°C. After an incubation of 15 min, the reaction was stopped with 6 ml dichloromethane and metabolites were extracted for HPLC analysis. 11β-OHT was used as an internal standard.

Glucuronyl transferase (GT)

The activity of hepatic UDP-glucuronyl transferase was determined in activated microsomes using 4-chlorophenol as a substrate for GT1 and 4-hydroxybiphenyl as a substrate for GT2 activity [18] according to the method of Mulder and Van Doorn [19] and adapted for a Cobas-Bio centrifugal analyser.

Glutathione S-transferase (GST)

The activity of hepatic and intestinal cytosolic GST was determined with 1-chloro-2,4-dinitrobenzene (5 µM) as a substrate using the spectrophotometric method of Habig *et al.* [20] and adapted for a Cobas-Bio centrifugal analyser.

DT-diaphorase (DTD)

Hepatic and intestinal cytosolic DTD activity was determined according to the method of Ernster [21] on a Cobas-Bio centrifugal analyser. Incubations were carried out in 320 µl Tris buffer (25 mM, pH 7.5) containing 575 µM NADH, 6.5 µM FAD, 0.22% Tween 20, 0.08% BSA, 1-8 µg cytosolic protein and 65 µM 2,6-dichlorophenol-indophenol as the substrate. The decrease in absorbance was followed at 600 nm for 3 min with and without 10 µM dicumarol.

Gel electrophoresis and immunoblotting

Microsomal proteins were separated by sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE) and immunoblotted as described previously [22]. The immunoblots were quantified using an ultrascan laser densitometer (LKB) and the results for each sample expressed as arbitrary optical density units per 2 µg microsomal protein. Liver microsomes prepared from rats treated with β-naphthoflavone, phenobarbital and dexamethasone (for preparation see [22]) were used as positive controls.

Statistical analysis

Data are presented as means ± SD where appropriate. Statistical differences were determined by one-way analysis of variance, followed by Dunnetts' multiple-comparison test. $P \leq 0.05$ was chosen as indicating significance.

Results

Body weights and food consumption

Body weights and food consumption did not differ significantly among the groups after 2, 7, and 14 days of dietary exposure to cooked Brussels sprouts (Table 2). After 28 days the 20%-sprouts group had a significantly lower body weight than the other groups. Food consumption appeared to be higher in the 20%-sprouts group, though the difference was only significant after one week of exposure. No differences were found in relative liver weight (data not shown).

Table 2. Mean values of body weight and food consumption of rats fed cooked Brussels sprouts.

Duration of Exposure (days)	0%	2.5%	5%	20%
	Body weight (g)[$]			
0	117.9 ± 2.9	117.9 ± 2.9	117.9 ± 2.9	117.9 ± 2.9
2	137.8 ± 13.2	137.0 ± 14.9	137.2 ± 16.1	130.0 ± 14.9
7	166.1 ± 10.1	165.3 ± 12.3	164.7 ± 18.5	164.9 ± 15.2
14	203.2 ± 9.3	198.0 ± 13.4	189.5 ± 15.9	194.0 ± 6.2
28	276.5 ± 18.4	259.5 ± 19.4	253.0 ± 25.7	229.5 ± 4.3*
	Food consumption (g/rat/day)[$$]			
2	27.9	30.9	31.3	32.1
7	30.1 ± 2.3	30.8 ± 2.5	31.9 ± 2.2	37.4 ± 2.1*
14	34.8 ± 3.4	34.5 ± 2.6	34.4 ± 2.5	38.7 ± 2.6
28	39.6 ± 3.6	37.6 ± 3.8	37.8 ± 2.9	41.9 ± 2.8

[$]Body weight values are means ± SD for groups of six animals (day 0: 24 animals). The values marked with asterisks differ significantly (ANOVA + Dunnett's test) from the control (0%) diet at the same time (* $P \leq 0.05$). [$$]The mean values for food consumption were recorded daily per cage of six animals.

Cytochrome P450-dependent activities in liver and small intestine

The hepatic EROD and PROD activities were both enhanced after 2 days of exposure in the 20%-sprouts group (Fig. 1; 1.3- and 2.5-fold, respectively), whereas after 14 and 28 days of exposure these activities were also enhanced in the 5%-sprouts group (both 1.6-fold). The 6β-hydroxylation activity towards testosterone was enhanced significantly in livers from rats treated with 20% cooked Brussels sprouts after 14 and 28 days of exposure (1.5- and 1.7-fold; Table 3). A decrease of the 2α-hydroxylation of testosterone to 70% of the activity in control rats was determined in liver from rats treated with 20% cooked Brussels sprouts after 7, 14 and 28 days of exposure. No differences were detected in the hydroxylation of testosterone at the 7α, 16α and 16β site (Table 3).

The intestinal EROD and PROD activities (Fig. 2) enhanced with increasing dietary levels of Brussels sprouts, but - due to marked inter-individual differences - were only significantly different from the control in the 20%-sprouts group after 2 and 28 days of exposure for the EROD activity (4.1- and 5.6-fold, respectively) and after 7 and 28 days of exposure for the PROD activity (7.5- and 7.2-fold, respectively). The 16β- and 16α-hydroxylation of testosterone were also increased (2.5- and 2.6-fold) after 2 days of exposure to 2.5% cooked Brussels sprouts (Table 4). A higher dose of sprouts enhanced the 16β- and 16α-hydroxylation of testosterone to *ca* 5.3-fold at day 7. After 28 days, however, these activities were only enhanced in the 20%-sprouts group. The intestinal 6β-hydroxylation of testosterone did not differ among the groups. The formation of 7α- and 2α-OHT in intestinal microsomes was below detection levels (Table 4).

SDS-PAGE and immunoblotting

Western blot analyses were carried out to ascertain whether the observed induction in P450-dependent catalytic activities were accompanied by elevation of specific P450 apoproteins. Indeed, from two days of exposure to cooked Brussels sprouts the hepatic P450 1A2 apoprotein level was markedly induced from 10 to 80 arbitrary optical density units (O.D.) (Fig. 3). The hepatic P450 1A1 (Fig. 3) and 2B1/2 apoprotein levels, however, were only induced in the 20% sprouts group from 10 to 40 and 30 O.D. units, respectively. In the liver of sprouts-fed animals no enhanced P450 3A apoprotein could be detected.

From two days, cytochrome P450 2B1/2 apoprotein was dose-relatedly induced in the mucosa of the small intestine of rats exposed to 2.5%, 5% and 20% sprouts to 13, 20 and 71 O.D. units, respectively (Fig. 4). After 14 days of exposure also low amounts of P450 1A1 (20 O.D. units) could be detected in the intestine of the 20%-sprouts group. The monoclonal antibody towards P450 3A cross-reacted with two other proteins in the small intestine, but the molecular weight of these proteins were both higher than the cytochrome P450 3A apoprotein in liver microsomes of dexamethasone-treated rats (Fig. 5). For both proteins a dose-related induction could be revealed.

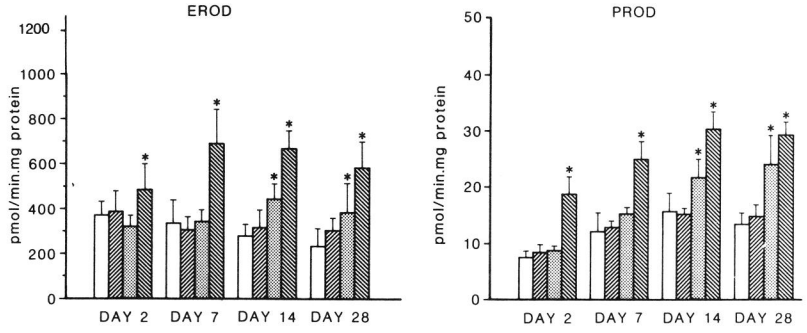

Fig. 1. 7-Ethoxyresorufin O-deethylation (EROD) and 7-pentoxyresorufin O-dependentylation (PROD) activities in **liver** microsomes of rats fed cooked Brussels sprouts for 2, 7, 14 and 28 days. □, 0% ; ▨, 2.5%; ⬚, 5%; ▧, 20% cooked Brussels sprouts. Values marked with asterisks differ significantly (ANOVA + Dunnetts' test) from the corresponding control value (* $P \leq 0.05$).

Table 3. The effects of dietary exposure to cooked Brussels sprouts on the P450 content and P450-dependent hydroxylation of testosterone in rat **liver**.

Day	Diet	P450 (pmol/mg protein)	6β-OHT	7α-OHT	16α-OHT (pmol/min/mg protein)	16β-OHT	2α-OHT
2	contr	850 ± 60	1069 ± 306	402 ± 94	2216 ± 658	94 ± 31	1317 ± 400
	2.5%	860 ± 90	958 ± 201	419 ± 94	2421 ± 1248	93 ± 31	1428 ± 771
	5%	830 ± 70	949 ± 205	414 ± 129	1973 ± 1051	84 ± 14	1180 ± 656
	20%	1010 ± 80*	1400 ± 209	448 ± 97	2095 ± 473	103 ± 15	1180 ± 318
7	contr	1060 ± 190	1120 ± 366	403 ± 120	5600 ± 1488	193 ± 78	3456 ± 871
	2.5%	950 ± 80	963 ± 100	305 ± 41	3754 ± 525	129 ± 19	2305 ± 319
	5%	980 ± 50	853 ± 165	330 ± 97	3361 ± 933	115 ± 36	2079 ± 562
	20%	1390 ± 180	1566 ± 213	420 ± 70	4092 ± 225	179 ± 14	2444 ± 167*
14	contr	1080 ± 70	812 ± 172	321 ± 94	4939 ± 925	197 ± 82	3113 ± 511
	2.5%	990 ± 90	899 ± 159	306 ± 87	4110 ± 762	157 ± 35	2564 ± 423
	5%	1110 ± 130	866 ± 237	390 ± 86	3741 ± 1781	133 ± 79	2335 ± 1047
	20%	1270 ± 160*	1234 ± 118*	332 ± 778	3735 ± 779	147 ± 34	2252 ± 450*
28	contr	1070 ± 50	775 ± 148	291 ± 47	4907 ± 569	170 ± 39	2995 ± 292
	2.5%	1150 ± 100	1038 ± 176	279 ± 21	5394 ± 838	187 ± 42	3232 ± 476
	5%	1210 ± 190	1096 ± 227	326 ± 64	5659 ± 1293	216 ± 50	3382 ± 759
	20%	1240 ± 120	1359 ± 273*	350 ± 53	3790 ± 1037	176 ± 57	2217 ± 589*

Values are means ± SD for six determinations. Those marked with asterisks differ significantly (ANOVA + Dunnetts' test) from the corresponding control value (* $P \leq 0.05$).

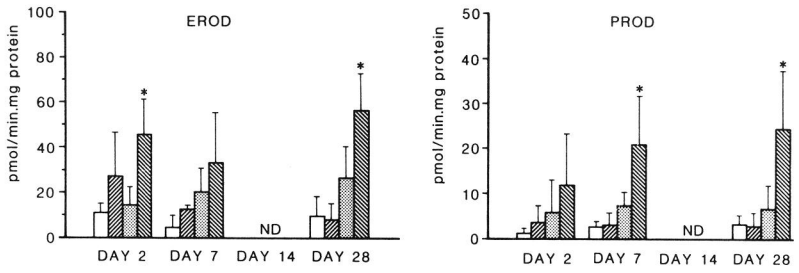

Fig. 2. 7-Ethoxyresorufin O-deethylation (EROD) and 7-pentoxyresorufin O-depentylation (PROD) activities in microsomes prepared from the **small intestine** of rats fed cooked Brussels sprouts for 2, 7, and 28 days. □, 0%; ▨, 2.5%; ▩, 5%; ▧, 20% cooked Brussels sprouts. Values marked with asterisks differ significantly (ANOVA + Dunnetts' test) from the corresponding control value (* $P \leq 0.05$).

Table 4. The effects of dietary exposure to cooked Brussels sprouts on the P450-dependent hydroxylation of testosterone, glutathione S-transferase and DT-diaphorase in the rat **small intestine.**

Day	Diet	6β-OHT	16α-OHT	16β-OHT	GST	DTD
		(pmol/min/mg protein)			(nmol/min. mg protein)	(nmol/min. mg protein)
2	contr	171	51	26	401 ± 108	107 ± 45
	2.5%	174	128	68	487 ± 101	180 ± 101
	5%	222	186	105	495 ± 146	272 ± 111*
	20%	156	211	114	823 ± 89*	341 ± 95*
7	contr	146	53	29	582 ± 163	67 ± 24
	2.5%	180	75	39	744 ± 179	91 ± 7
	5%	189	141	73	776 ± 58	133 ± 85
	20%	176	277	157	1053 ± 157*	229 ± 209
28	contr	176	68	47	694 ± 116	140 ± 55
	2.5%	149	77	37	581 ± 141	183 ± 156
	5%	161	90	44	707 ± 86	150 ± 54
	20%	120	267	144	1022 ± 162*	212 ± 129

Values are means of duplicate measurements for the hydroxylation of testosterone and means ± SD for six determinations for GST and DTD. Those marked with asterisks differ significantly (ANOVA + Dunnett's test) from the corresponding control value (* $P \leq 0.05$).

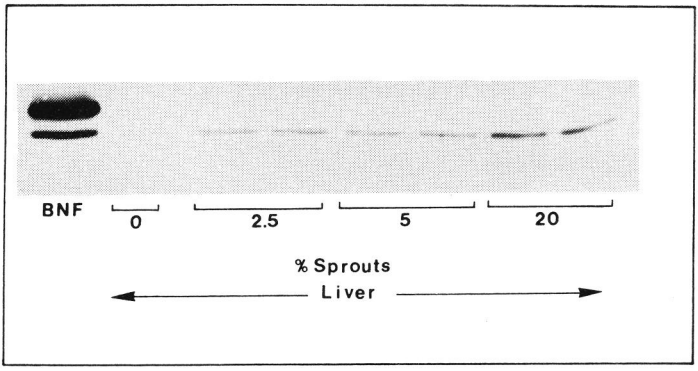

Fig. 3. SDS-PAGE immunodetection of P450 1A1/2 apoproteins in liver microsomes prepared from rats treated with 0%, 2.5%, 5% and 20% cooked Brussels sprouts. Liver microsomes prepared from a rat treated with β-naphthoflavone (BNF) were used as a positive control. The immunoblots were quantified with laser densitometry and results are given in arbitrary optical density (O.D.) units. Upper band: P450 1A1: 20%, 39 O.D.; BNF, 865 O.D. Lower band: P450 1A2: 0%, 10 O.D.; 2.5%, 31 O.D.; 5%, 31 O.D.; 20 %, 79 O.D.; BNF, 278 O.D. (Values are means of duplicate measurements).

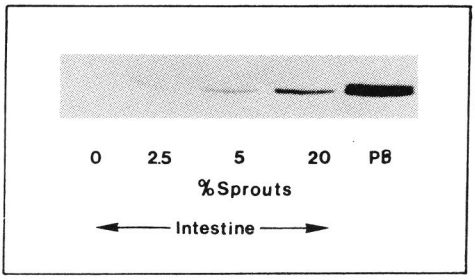

Fig. 4. SDS-PAGE-immunodetection of P450 2B1/2 apoproteins in microsomes prepared from the small intestine of rats treated with 0%, 2.5%, 5% and 20% cooked Brussels sprouts. Liver microsomes prepared from a rat treated with phenobarbital (PB) were used as a positive control. The immunoblots were quantified with laser densitometry and results are given in arbitrary optical density (O.D.) units. P450 1B1: 2.5%, 13 O.D.; 5%, 20 O.D.; 20%, 71 O.D.; PB, 217 O.D.

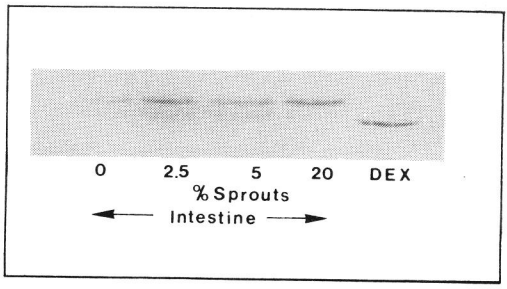

Fig. 5. SDS-PAGE-immunodetection of P450 3A apoprotein in microsomes prepared from the small intestine of rats treated with 0%, 2.5%, 5% and 20% cooked Brussels sprouts. Liver microsomes prepared from a rat treated with dexamethasone (DEX) were used as a positive control. Immunoblots were quantified with laser densitometry and results are given in arbitrary optical density units (O.D.). Upper band: 0%, 27 O.D.; 2.5%, 30 O.D.; 5%, 48 O.D.; 20%, 46 O.D. Lower band: 0%, 18 O.D.; 2.5%, 18 O.D.; 5%, 20 O.D.; 20%, 26 O.D. DEX microsomes: P450 3A, 68 O.D.

Phase II enzymes in liver and intestine

The effects of dietary Brussels sprouts on GST, GT1, GT2 and DTD in the liver are given in Table 5. GST activity was increased after 2 days of exposure to 5% and 20% cooked Brussels sprouts (1.2-fold). This difference increased to 1.5-fold throughout the experiment. The GT1 activity was increased in the 20%-sprouts group after 2 (1.2-fold), 7 (1.6-fold), 14 (1.9-fold) and 28 (2.4-fold) days of exposure. After 28 days, the GT1 activity was also increased in the 2.5%- and the 5%-sprouts group (*ca* 1.4-fold). A decrease to *ca* 75% of the control group in GT2 activity was detected in livers from rats treated with 2.5% and 5% cooked Brussels sprouts during the first week of exposure. In the 20%-sprouts group, however, the GT2 activity was higher than in the control group at day 14 and 28. The DTD activity in the liver was enhanced in rats fed with 20% Brussels sprouts after 2 (1.9-fold) and 28 days (3.5-fold; Table 5).

Intestinal GST activity was significantly increased in rats treated with 20% cooked Brussels sprouts after 2, 7 and 28 days of exposure to 2.1-, 1.8- and 1.5-fold, respectively. Dietary exposure to lower levels of cooked Brussels sprouts did not affect the intestinal GST activity (Table 4). The DTD activity in the small intestine was enhanced at a dose of 5% and 20% cooked Brussels sprouts after two days of exposure (2.5- and 3.2-fold, respectively; Table 4). Longer exposure to these diets resulted in enhanced DTD activities, but the difference towards the control group diminished after 28 days.

Table 5. The effects of dietary exposure to cooked Brussels sprouts on glutathione S-transferase, glucuronyl transferase and DT-diaphorase activities in rat **liver.**

Day	Diet	GST (μmol/min. mg protein)	GT1[$] (nmol/min. mg protein)	GT2[$] (nmol/min. mg protein)	DTD (nmol/min. mg protein)
2	contr	2.6 ± 0.3	96 ± 10	37 ± 3	845 ± 432
	2.5%	2.8 ± 0.4	101 ± 13	32 ± 2*	858 ± 452
	5%	3.0 ± 0.3*	110 ± 12	39 ± 4	1373 ± 723
	20%	3.0 ± 0.3*	117 ± 4*	39 ± 4	1581 ± 311*
7	contr	2.0 ± 0.2	82 ± 14	38 ± 8	704 ± 388
	2.5%	2.2 ± 0.2	74 ± 7	29 ± 3*	500 ± 329
	5%	2.4 ± 0.2*	74 ± 10	29 ± 2*	831 ± 487
	20%	3.0 ± 0.3*	131 ± 27*	46 ± 9	673 ± 553
14	contr	2.1 ± 0.3	43 ± 8	23 ± 4	411 ± 353
	2.5%	2.3 ± 0.2	49 ± 6	24 ± 4	292 ± 198
	5%	2.2 ± 0.2	51 ± 9	28 ± 3	440 ± 439
	20%	3.1 ± 0.2*	80 ± 12*	32 ± 4*	574 ± 383
28	contr	1.9 ± 0.2	30 ± 4	19 ± 4	443 ± 272
	2.5%	1.9 ± 0.2	40 ± 3*	22 ± 3	534 ± 163
	5%	2.3 ± 0.1*	47 ± 7*	24 ± 6	870 ± 264
	20%	3.0 ± 0.2*	73 ± 7*	30 ± 2*	1556 ± 529*

Values are means ± SD for six determinations. Those marked with asterisks differ significantly (ANOVA + Dunnetts' test) from the corresponding control value (* $P \leq 0.05$). [$] glucuronyl transferase activity was measured towards 4-chlorophenol (GT1) and 4-hydroxybiphenyl (GT2).

Discussion

We have shown that dietary exposure to cooked Brussels sprouts at levels as low as 2.5 and 5% in the diet for only two days can result in markedly induced levels of P450 2B1 in the small intestine and P450 1A2 in the liver. Besides immunochemical detection of apoproteins, we used marker substrates to study the catalytic activities of these and some other forms of P450. In this respect, the selective dealkylation of ethoxy- and pentoxyresorufin are used as marker activities for the P450 1A1/2 and P450 2B1/2 enzymes, respectively [16]. The regioselective hydroxylation of testosterone provides the catalytic activities of four P450 enzymes i.e. the formation of 2α-OHT is predominantly catalysed by P450 2C11, 16β-OHT by P450 2B1 [23,24], 7α-OHT by P450 2A [25] and 6β-OHT by P450 3A [26]. In our study, the enhanced EROD activity in the liver from rats exposed to 5% and 20% sprouts corresponds with the higher P450 1A2 and 1A1

apoprotein levels. The induction of this P450 1A subfamily by both cruciferous vegetables and isolated indoles as measured as arylhydrocarbon hydroxylase activity or EROD was already suggested by others [5,6]. The higher PROD activity, however, was not accompanied by enhanced P450 2B apoprotein levels in the liver. Nakajima *et al.* [27] reported that in untreated animals the more constitutive enzyme P450 2C11 is highly responsible for the EROD and PROD activities in absence of P450 1A1 and P450 2B1, respectively, indicating that at a low rate these activities are not that specific as generally is assumed. Moreover, it has been shown that in liver microsomes from BNF-treated rats the PROD activity is co-enhanced to 5-fold without induction of P450 2B apoprotein levels [22]. The higher formation of 6β-OHT in the liver of the 20% sprouts group is probably also due to other P450-forms than P450 3A (e.g. P450 1A [26]), because no induction of this P450 apoprotein could be detected. The decreased hydroxylation rate at the 2α site of testosterone in the 20% sprouts group indicates a reduction of P450 2C11, a more constitutive P450 enzyme [26]. A similar coordinated decrease of 2α-OHT in the liver has been reported for other P450 1A1/2 inducers [22,24]. The fact that the formation of 7α-OHT is not influenced by Brussels sprouts, suggests the absence of an effect of these diets on P450 2A in the liver [25].

The induced P450 2B apoprotein level in the small intestine was mirrored by enhanced PROD and formation of 16β-OHT and 16α-OHT, both preferentially catalysed by P450 2B [23]. The higher induction ratio for both EROD and PROD in the small intestine as compared to the induction ratio in the liver is in agreement with previous data from our own laboratory [28] and data reported by others [4,5]. In the present study, however, only very low apoprotein levels of P450 1A1 and 1A2 could be detected in the small intestine. The induction and immunochemical detection of both P450 1A1 as well as P450 2B1 and P450 3A apoproteins in the small intestinal mucosa of rats due to oral exposure to various xenobiotics and dietary components has been described [29-31]. Moreover, Lindeskog *et al.* [29] described almost complete inhibition of EROD activity in the small intestine from untreated and BNF-treated animals with antibodies raised towards P450 1A1. Whether the 8-fold induction of EROD activity in the small intestine in our study is related to the highly enhanced P450 2B or the only slightly enhanced P450 1A apoprotein (or even other forms of P450) is not clear. A broader range of substrate specificities for P450 enzymes in extrahepatic tissues than in liver was also suggested by Sesardic *et al.* [32] who reported P450 1A1-immunoinhibition of the O-deethylation of phenacetin in small intestine, whereas no catalytic activity of P450 1A1 towards phenacetin in the liver could be detected. These data emphasize the need to determine both catalytic activities and apoprotein and/or mRNA levels to determine which P450 enzyme could be responsible for the observed changes in metabolic profile.

Along with the enhancement of both P450 1A and P450 2B, the activity of GST and DTD were increased in both liver and small intestine, after a period as short as two days to 5% cooked Brussels sprouts, already. Previously, Bogaards *et al.* [10] reported a linear relationship between the amount of Brussels sprouts and the induction of different subunits of GST, after an exposure of *ca* 4 weeks. In the present study we show a time-related induction of both GST, GT and DTD in the liver. The fact that a third phase II enzyme, GT1, was also enhanced in the liver as a result of dietary exposure to cooked

Brussels sprouts, indicates even more the wide spectrum of enhanced biotransformation pathways after consuming this type of cruciferous vegetable.

In humans frequent ingestion of cruciferous vegetables is associated with a decrease in risk for cancer in the gastro-intestinal tract [33,34]. Feeding animals diets containing either cruciferous vegetables or isolated glucosinolate-breakdown products decreased the tumorigenesis associated with e.g. aflatoxin B_1 and dimethylbenz[a]anthracene [35,36]. The inhibition of carcinogenesis by e.g. antioxidants is highly associated with the induction of phase II enzyme GST (for review see Williams *et al.*, 1989). On the other hand, the markedly enhanced cytochrome P450 catalytic activities in the small intestine may result in increased intestinal first pass clearance of xenobiotics - which has been shown the case for the drug phenacetin [37] - but also for various food compounds co-ingested within one single meal. In this respect, McDanell *et al.* [38] reported a marked induction of P450 activity (EROD) in the small intestine of rats within 4 to 6 hr after a single meal of 25% Brussels sprouts.

The present study has shown the induction of both phase I and phase II enzymes after only a short exposure to Brussels sprouts. The disturbance in biotransformation activities in the liver but especially in the small intestine could have great consequences for the metabolism of various biologically active compounds.

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CHAPTER 5

EFFECTS OF INDOLE-3-CARBINOL ON BIOTRANSFORMATION ENZYMES IN THE RAT

In vivo changes in liver and small intestinal mucosa in comparison with primary hepatocyte cultures

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Abstract

Groups of male Wistar rats were fed semi-synthetic diets containing 0, 200 and 500 mg/kg indole-3-carbinol (I3C) for either 2, 7, 14 or 28 days. Two days of exposure I3C already induced P450 enzymes, but the enzyme pattern induced were different in the liver and small intestine. In the liver P450 1A1, 1A2 and 2B1 apoprotein levels were dose-relatedly enhanced, whereas in the small intestine induced levels of P450 2B1, 1A1 but not 1A2 apoprotein were detected. Pentoxy- and ethoxyresorufin dealkylation (PROD and EROD) were dose-relatedly enhanced in liver (5- and 7-fold, respectively) as well as small intestine (8- and 13-fold, respectively) after two days. Testosterone 16α- and 16β-hydroxylation in the small intestine were enhanced (6 to 9-fold) from two days onwards, whereas in the liver these activities were only slightly enhanced from 7 days onwards. Thus, the major forms induced in the liver appear to be P450 1A1, 1A2 and 2B1, whereas in small intestine all effects found are associated with a single P450, namely 2B1.

After two days I3C (500 mg/kg) slightly induced glutathione S-transferase in liver and small intestine. Hepatic glucuronyl transferase (GT1) was induced (*ca.* 1.6-fold) after 7, 14 and 28 days. DT-diaphorase in liver and small intestine was induced from 14 days of exposure to 500 mg/kg I3C (2.7- and 1.5-fold, respectively).

Treatment of rat hepatocytes with indole-3-acetonitrile and 3,3'-diindolylmethane, but not I3C and indole-3-carboxaldehyde, enhanced the EROD activity and halved testosterone 16α- and 2α-hydroxylation. All four indoles slightly induced glutathione S-transferase in cultured hepatocytes. Thus, the *in vitro* studies suggest that the *in vivo* effects of I3C have to be attributed to indole-condensation products, such as 3,3'-diindolylmethane, but not to I3C itself.

Fd Chem Toxic (submitted)

Introduction

The indolylmethyl glucosinolate (glucobrassicin) is a major compound found in cruciferous vegetables such as broccoli, Brussels sprouts, cabbage and cauliflower [1]. Disruption of cells and hydrolysis of glucobrassicin by the plant enzyme myrosinase results in the formation of several indole compounds, among them indole-3-carbinol (I3C), indole-3-acetonitrile, and 3,3'-diindolylmethane [2,3]. Increased intake of cruciferous vegetables is associated with a decreased risk of cancer of the colon, rectum and bladder in man [4]. Feeding rodents a diet supplemented with brassica vegetables or isolated indole glucosinolate hydrolysis products decreased e.g. aflatoxin B_1-induced liver neoplasia, dimethylbenz[a]anthracene-induced mammary tumorigenesis and benzo[*a*]pyrene-induced forestomach and lung tumour formation (for review see [3]). This inhibitory effect towards a wide range of carcinogens is thought to be associated with an overall inductive effect on several biotransformation enzymes [5].

Cruciferous vegetables are effective inducers of both phase I (cytochromes P450) and phase II enzymes in rodents and humans [3,6]. Dietary exposure to I3C alone results also in an enhancement of phase I and - to a lesser extent - phase II enzyme activities in the liver and, more pronounced, in the small intestine of rodents [7-10]. So far, in most studies the cytochrome P450 activities associated with the subfamily P450 1A were determined. Now, more and more is known about the diversity of the superfamily of P450 enzymes, among which are four different xenobiotic-inducible families [11]. In a previous study, we studied the P450 pattern in the liver and the small intestine of rats fed cooked Brussels sprouts [12]. P450 1A2 was the predominant form to be induced in the liver, whereas in the small intestine P450 2B1 was induced. Moreover, a decreased hepatic hydroxylation of testosterone at the 2α-site was measured, indicating a decreased level of the constitutive P450-form, P450 2C11 [13]. This latter enzyme is associated with the formation of the reactive metabolite of aflatoxin B_1 in rat [14]. On the other hand, induction of phase II enzymes e.g. glutathione S-transferase (GST; EC 2.5.18) and DT-diaphorase (DTD; NAD(P)H:quinone oxidoreductase; EC 1.6.99.2) is also correlated with a decrease in tumorigenesis [5,15].

The aim of the present study was to investigate the effects of I3C on the cytochrome P450 profile in liver and small intestine in more detail. Different marker substrates and Western blotting were used to determine the catalytic activities and apoprotein levels of P450 enzymes. In addition, the effects of I3C on several phase II enzymes (GST, DTD and glucuronyl transferases (GT; EC. 2.4.1.17)) were determined. To evaluate which indole-derivative could be responsible for the changes in biotransformation activities primary cultures of rat hepatocytes were treated with I3C, indole-3-acetonitrile (I3A), 3,3'-diindolylmethane (DIM) and indole-3-carboxaldehyde (I3CA, an oxidative metabolite of both I3C and I3A (Van Bladeren, unpublished results)).

Materials and methods

Chemicals

Indole-3-carbinol was obtained from Aldrich Chemical Co., Milwaukee, WI, USA. Testosterone, 11β- and 16α-hydroxytestosterone (11β- and 16α-OHT) were obtained from Sigma Chemical Co., St Louis, MO, USA. 6β-, 7α-, 16β- and 19-OHT were obtained from Steraloids, Wilton, NH, USA. 2α-OHT was a kind gift from Prof D.N. Kirk (Queen Mary College, University of London), 15β-OHT was a gift from G.D. Searle and Co., Skokie, IL, USA. 3,3'-diindolylmethane was a kind gift from Ir J. Vuik (TNO-Toxicology and Nutrition Institute, Zeist, The Netherlands). All other chemicals were of analytical grade. Monoclonal antibodies directed towards P450 1A1/2, P450 2B1/2 and P450 3A were kind gifts from Dr P.J. Kremers, Université de Liège, Belgium. Polyclonal antibodies directed towards P450 2E and P450 4A were kind gifts from Dr I. Johansson (Karolinska Institutet, Stockholm, Sweden) and Dr G.G. Gibson, University of Surrey, Guildford, UK), respectively. Secondary antibodies (alkaline phosphatase labeled) were obtained from Dakopatts a/s, Glostrup, Denmark.

Animals and diet

Male Wistar rats (Crl:(WI)Br), aged 8 wk, were obtained from Charles River WIGA (Sulzfeld, FRG). Animals were housed in groups of five in stainless steel cages with wire-screen bottom and front. The room temperature was maintained at 21 ± 2°C and the relative humidity at 40-70%. A light/dark cycle of 12 hr was maintained and the ventilation rate was *ca.* ten air changes/hr. Food and water were available *ad libitum.* Before the experiment all rats were fed a TNO open-formula basal diet for 7 days. Rats were allocated to three diet groups of 15 animals using a computer-generated random-number table.

Indole-3-carbinol was incorporated into a semi-synthetic diet at levels providing 200 and 500 mg/kg diet. The composition of the test diets are given in Table 1. Diets were kept refrigerated at 3°C until new diets were prepared (every three days). Body weights of the individual rats were recorded weekly. Food intake was measured daily. After 2, 7, 14 and 28 days, respectively, rats were killed by bleeding from the aorta abdominalis under ether anaesthesia. The livers and the proximal 20 cm of the small intestines were collected.

Preparation of microsomes and cytosols

All procedures were performed at temperatures ranging from 0-4°C. Microsomes and cytosol of liver were prepared according to Rutten *et al* [16]. Livers were homogenized in 3 volumes ice-cold Tris-buffered 1.15% KCl (0.01 M Tris-HCl, pH 7.4) using a Potter-Elvehjem glass-Teflon homogenizer. After centrifugation for 15 min at 9000 ***g***, the post-mitochondrial fraction was centrifuged for 60 min at 105,000 ***g***. The microsomal pellet was resuspended in Tris buffered 1.15% KCl with an ultra-turrax. Cytosol and microsomal fractions were quickly frozen in liquid N_2 and stored at -80°C. Microsomes and cytosol of the small intestine were prepared according to Borm *et al* [17] with modifications as described by Bogaards *et al* [18]. Briefly, the proximal 20 cm

of the small intestine was perfused with ice-cold phosphate-buffered saline (PBS) and subsequently slipped inside out over 3-mm diameter stainless steel pins mounted on a Vibro-mixer. The epithelial cells were released by vibration in PBS containing 5 mM EDTA for 40 min. The cells were repeatedly washed, suspended in 2 ml PBS and sonicated for 10 sec (amplitude 6 μm). After centrifugation at 2500 ***g*** for 20 min, the post-mitochondrial fraction was centrifuged at 105.000 ***g*** for 60 min. The microsomal pellet was resuspended in 1 ml PBS with an ultra turrax. Microsomes and cytosol were quickly frozen in liquid N_2 and stored at -80°C.

Table 1. Composition of the diets

Ingredients	Concentration (g/kg diet) Indole-3-carbinol		
	Control	200 mg/kg	500 mg/kg
Casein	130	130	130
Cellulose	50	50	50
Mineral mix*	35	35	35
Vitamin ADEK prep.**	3	3	3
Vitamin B mixture***	2	2	2
Corn-oil	50	50	50
Wheat starch (native)	300	300	300
Soy-protein isolate	130	130	130
Sucrose	300	300	300
Indole-3-carbinol	0	0.2	0.5

* The mineral mixture contained (per gram mix): 399 mg KH_2PO_4, 389 mg $CaCO_3$, 142 mg NaCl, 58 mg $MgSO_4$, 5.7 mg $FeSO_4.7H_2O$, 0.9 mg $ZnCl_2$, 0.8 mg $CuSO_4.5H_2O$, 4.6 mg $MnSO_4.2H_2O$, 0.02 mg $CoCl_2.6H_2O$, 0.08 mg $KCr(SO_4)_2.12H_2O$.

** The ADEK vitamin preparation contained (per gram): 939 mg vitamin AD concentrate, containing 2 IU/g vitamin A and 750 IU/g vitamin D, 15 mg vitamin E, 1.0 mg vitamin K_3, 45 mg wheat starch.

*** The vitamin B mixture contained (per gram): 2.5 mg thiamine-HCl, 3.0 mg riboflavin, 10 mg pyridoxin-HCl, 12.5 mg niacin, 7.5 mg Ca-pantothenate, 0.075 mg biotin, 0.5 mg folic acid, 0.035 mg vitamin B_{12}, 964 mg ground sucrose.

Isolation and culture of hepatocytes

Hepatocytes were isolated from untreated rats and cultured as described previously [19]. After 24 hr in culture, indoles dissolved in dimethyl sulfoxide (DMSO) were added to the culture media. The highest non-cytotoxic concentration was used (10 μg/ml of I3C

(68 nM), I3CA (69 nM) and DIM (36 nM), respectively and 40 μg/ml I3A (260 nM)). An equal amount of DMSO was added to the control cultures (final DMSO concentration 0.1% v/v). Media with/without indoles were refreshed every 24 hr. After 48 hr of exposure, cells were harvested, and microsomes and cytosol prepared as described [19].

Enzyme assays

Cytochrome P450 and protein

Contents of protein and total hepatic cytochrome P450 were determined according to Rutten *et al* [16].

Ethoxy- and pentoxyresorufin O-dealkylation (EROD and PROD)

Fluorimetric determinations of the dealkylation of ethoxyresorufin (EROD) and pentoxyresorufin (PROD) were performed according to the method of Burke *et al* [20] using a Cobas-Bio centrifugal analyser equipped with a spectrofluorimeter. Less than 200 μg microsomal protein was used in an incubation mixture of 0.1 M phosphate buffer (pH 7.4) and NADPH regenerating system in a final volume of 320 μl. Substrate (final concentration 5 μM) was added as diluted in DMSO (final DMSO concentration 0.5% v/v).

Testosterone hydroxylation

Hepatic and intestinal hydroxylation of testosterone was determined as described by Wortelboer *et al* [19]. In short, microsomes (150-400 μg protein) were incubated in 1 ml potassium-phosphate buffer (50 mM, pH 7.4) containing 3 mM $MgCl_2$, 1 mM EDTA, 1 mM $NADP^+$, 5 mM glucose-6-phosphate, 1 unit/ml glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and 250 μM testosterone at 37°C. After an incubation of 15 min, the reaction was stopped with 6 ml dichloromethane and metabolites were extracted for HPLC analysis. 11β-OHT was used as an internal standard.

Glucuronyl transferases (GT)

The activity of GTs were determined in activated hepatic microsomes using 4-chlorophenol as a substrate for GT1 and 4-hydroxybiphenyl as a substrate for GT2 activity [21] according to the method of Mulder and Doorn [22] on a Cobas-Bio centrifugal analyser.

Glutathione S-transferase (GST)

The activity of cytosolic GST was determined with 1-chloro-2,4-dinitrobenzene (5 μM) as substrate using the spectrophotometric method of Habig *et al* [23] adapted for a Cobas-Bio centrifugal analyser.

DT-diaphorase (DTD)

DTD activity was determined according to the method of Ernster [24] on a Cobas-Bio centrifugal analyser. Incubations were carried out in 320 μl Tris buffer (25 mM, pH 7.5) containing 575 μM NADH, 6.5 μM FAD, 0.22% Tween 20, 0.08% BSA, 1-8 μg cytosolic protein and 65 μM 2,6-dichlorophenolindophenol as the substrate. The decrease in absorbance was followed at 600 nm for 3 min with and without 10 μM dicumarol.

Gel electrophoresis and Western blotting

Microsomal proteins (2 μg per lane) were separated using a discontinuous sodium dodecyl sulphate poly-acrylamide gel electrophoresis system and subsequently immunoblotted as described previously [19]. The immunoblots were quantified using an

Ultrascan Laser Densitometer (LKB) and the results for each sample are expressed as arbitrary optical density units. Liver microsomes prepared from rats treated with β-naphthoflavone, phenobarbital, dexamethasone, isoniazid and clofibrate were used as positive controls (for preparation see [19]).

Statistical analysis

Data are presented as means ± SD where appropriate. Statistical differences were determined by a one-way analysis of variance, followed by Dunnetts' multiple-comparison tests. $P \leq 0.05$ was chosen to indicate significance.

Results

Body weights and food consumption

Body weights and food consumption did not differ significantly among the groups after exposure to indole-3-carbinol (Table 2). No differences were found in relative liver weight (data not shown).

Table 2. Body weights and food consumption of rats fed indole-3-carbinol.

Duration of exposure (days)	Body weight (g)[$]			Food consumption (g/rat/day)[$$]		
	control	200 mg/kg	500 mg/kg	control	200 mg/kg	500 mg/kg
0	173.9 ± 2.6	174.2 ± 2.6	174.4 ± 2.7			
2	208.7 ± 15.5	209.3 ± 13.2	201.5 ± 9.7	ND	ND	ND
7	230.6 ± 18.9	239.8 ± 14.3	235.9 ± 17.3	22.3	21.7	20.3
14	288.2 ± 21.0	277.0 ± 21.1	271.8 ± 8.5	20.4	19.9	19.3
28	349.6 ± 35.4	353.0 ± 70.7	317.6 ± 34.1	22.2	21.7	21.2

[$]Body weight values are means ± SD for groups of five animals (day 0: 20 animals). [$$]The mean values for food consumption were recorded weekly per cage of five animals. ND = not determined.

Cytochrome P450-dependent activities in liver and small intestine

After 2, 7 and 14 days cytochrome P450 levels in the liver were *ca.* 1.4-fold enhanced in rats fed 500 mg I3C/kg diet (Table 3). After 28 days the cytochrome P450 content was similar in the livers from control rats and I3C-treated rats. The hepatic EROD and PROD activities were both enhanced (6.7- and 5.2-fold, respectively) after 2 days of exposure in rats treated with the highest dose I3C (Fig. 1). Longer dietary exposure to I3C enhanced the EROD activities also in the liver of rats fed 200 mg I3C/kg diet. The induction ratio (treated/control at the same day) for EROD diminished during the experiment (from 5.9 to 3.1-fold). A similar time-related decrease in induction ratio was observed for the PROD activity in livers from rats fed the high dose I3C (from 3.9 to 2.3-fold).

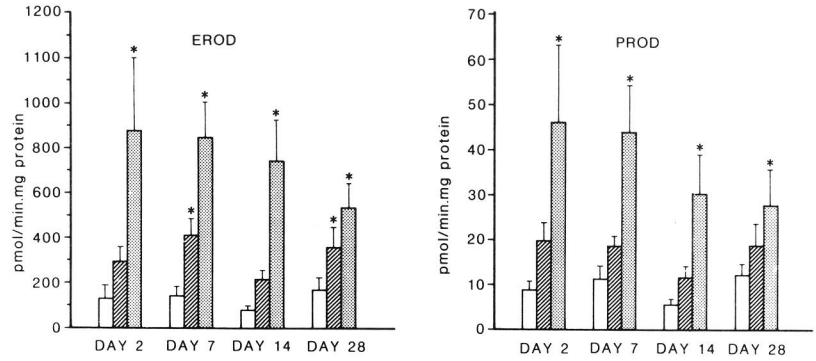

Fig. 1. The O-dealkylation of 7-ethoxyresorufin (EROD) and 7-pentoxyresorufin (PROD) activities in **liver** microsomes of rats fed indole-3-carbinol for 2, 7, 14 and 28 days. □, control ; ▨, 200 mg/kg diet; ▩, 500 mg/kg diet indole-3-carbinol. Values marked with asterisks differ significantly (ANOVA + Dunnett's test) from the corresponding control value (* $P \leq 0.05$).

Table 3. The effects of dietary exposure to indole-3-carbinol on P450 content and P450-dependent hydroxylation of testosterone in rat **liver**.

Day	I3C mg/kg	P450 (pmol/ mg protein)	6β-OHT	7α-OHT	16α-OHT	16β-OHT	2α-OHT
					(pmol/min/mg protein)		
2	0	600 ± 40	725 ± 285	440 ± 69	4599 ± 534	120 ± 35	2781 ± 299
	200	680 ± 90	969 ± 333	533 ± 77	4749 ± 1268	143 ± 67	2840 ± 740
	500	790 ± 80*	1227 ± 254*	502 ± 104	4899 ± 792	212 ± 84	2858 ± 398
7	0	560 ± 80	929 ± 131	425 ± 82	4210 ± 997	110 ± 43	2498 ± 591
	200	720 ± 70*	845 ± 307	372 ± 43	5991 ± 667	203 ± 42*	3514 ± 377*
	500	760 ± 100*	1336 ± 327	433 ± 53	5570 ± 1082	287 ± 79*	3158 ± 591
14	0	490 ± 90	606 ± 195	373 ± 45	3183 ± 772	86 ± 25	1919 ± 447
	200	630 ± 140	1024 ± 195*	381 ± 115	3766 ± 1070	121 ± 31	2197 ± 610
	500	690 ± 60*	1330 ± 325*	510 ± 73	4655 ± 1320	215 ± 53*	2576 ± 741
28	0	840 ± 120	1022 ± 395	476 ± 114	5055 ± 1932	143 ± 60	3053 ± 1012
	200	820 ± 120	1345 ± 699	468 ± 101	7141 ± 1845	307 ± 127	4125 ± 954
	500	850 ± 60	1080 ± 323	489 ± 33	6577 ± 1487	291 ± 106	3632 ± 740

Values are means ± SD for five determinations. Those marked with asterisks differ significantly (ANOVA + Dunnetts' test) from the corresponding control value (* $P \leq 0.05$).

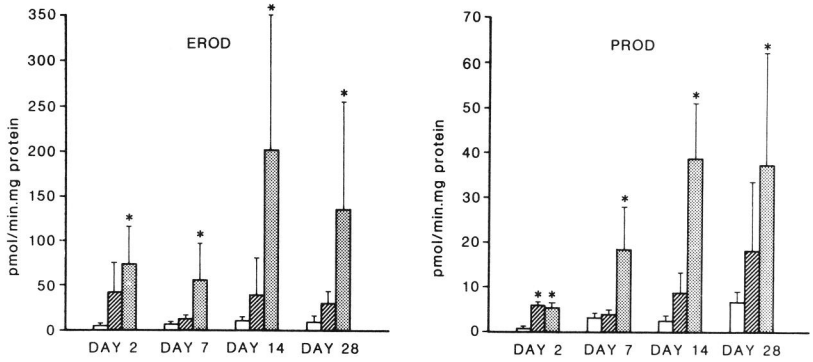

Fig. 2. The O-dealkylation of 7-ethoxyresorufin (EROD) and 7-pentoxyresorufin (PROD) activities in microsomes prepared from the **small intestine** of rats fed indole-3-carbinol for 2, 7, 14 and 28 days. ☐, control; ▨, 200 mg/kg diet; ▩, 500 mg/kg diet indole-3-carbinol. Values marked with asterisks differ significantly (ANOVA + Dunnett's test) from the corresponding control value (* $P \leq 0.05$).

Table 4. The effects of dietary exposure to indole-3-carbinol on P450-dependent activities, glutathione S-transferase and DT-diaphorase in rat **small intestine**.

Day	I3C mg/kg	6β-OHT	7α-OHT	16α-OHT	16β-OHT	GST	DTD
		(pmol/min/mg protein)				(nmol/min/ mg protein)	(nmol/min/ mg protein)
2	0	50 ± 14	39 ± 13	12 ± 5	7 ± 3	736 ± 187	69 ± 14
	200	86 ± 19	50 ± 16	113 ± 75*	63 ± 41*	780 ± 225	74 ± 28
	500	72 ± 31	60 ± 19	72 ± 40*	41 ± 23*	805 ± 99	76 ± 16
7	0	90 ± 71	64 ± 26	30 ± 12	17 ± 6	818 ± 135	73 ± 19
	200	56 ± 12	40 ± 7	37 ± 19	20 ± 8	807 ± 143	77 ± 23
	500	103 ± 74	66 ± 29	157 ± 116*	93 ± 68	874 ± 306	92 ± 22
14	0	74 ± 27	63 ± 16	14 ± 18	7 ± 8	807 ± 204	57 ± 5
	200	79 ± 37	48 ± 18	78 ± 60	44 ± 33	1011 ± 247	69 ± 15
	500	137 ± 40	78 ± 17	304 ± 87*	173 ± 49*	1309 ± 178*	80 ± 12*
28	0	134 ± 43	63 ± 28	42 ± 31	25 ± 20	860 ± 132	92 ± 29
	200	144 ± 50	84 ± 15	120 ± 45*	69 ± 27	1030 ± 202	84 ± 21
	500	89 ± 23	78 ± 10	179 ± 102*	108 ± 58*	1253 ± 201*	136 ± 811

Values are means ± SD of five determinations. Those marked with asterisks differ significantly (ANOVA + Dunnetts' test) from the corresponding control value (* $P \leq 0.05$).

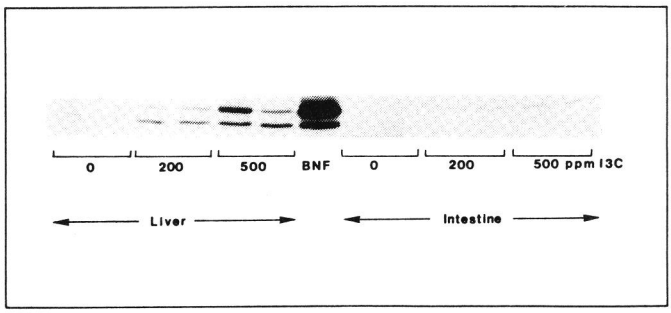

Fig. 3. SDS-PAGE-immunodetection of P450 1A1/2 apoproteins in liver and small intestinal microsomes prepared from rats treated with 0, 200 and 500 mg/kg diet indole-3-carbinol for two days. Liver microsomes prepared from a rat treated with ß-naphthoflavone (BNF) were used as a positive control. Immunoblots were quantified with laser densitometry and results of two individual rats are given in arbitrary optical density (O.D.) units. P450 1A1 (upper band): liver 0: < 5 O.D.; 200: 15/25 O.D.; 500: 222/53 O.D.; BNF: 863 O.D.; small intestine 0: < 5 O.D.; 200: 33/37 O.D.; 500: 19/13 O.D. P450 1A2 (lower band): liver 0: <5/8 O.D.; 200: 44/45 O.D.; 500: 86/103 O.D.; BNF: 278 O.D.; small intestine: not detectable.

Testosterone hydroxylation was enhanced at the 6β-site in the liver of rats exposed to 500 mg/kg I3C for 2 days (1.7-fold) and in both dose groups after 14 days (1.7- and 2.2-fold, respectively; Table 3). From one week of exposure the formation of 16β-OHT in liver microsomes from rats fed the highest dose was enhanced, too (to *ca.* 2.5-fold). No differences were detected in the hydroxylation of testosterone at the 7α, 16α and 2α site.

In the small intestine the EROD and PROD activities (Fig. 2) were markedly enhanced from two days onwards in rats exposed to the high dose of I3C (EROD range: 7.8 to 18.2-fold; PROD range 5.5 to 14.4-fold). After two days the intestinal 16α- and 16β-hydroxylation of testosterone were also increased even to a higher level in rats fed the low dose of I3C (both *ca.* 9-fold; Table 4) in comparison with rats fed the high dose (*ca.* 6-fold enhancement). The highest levels of both 16α- and 16β-hydroxylation of testosterone were determined after 14 days of exposure to 500 mg/kg diet I3C (22- and 25-fold). No differences were observed in intestinal 6β- and 7α-hydroxylation of testosterone. The formation of 2α-OHT in intestinal microsomes was below detection levels (Table 4).

SDS-PAGE and Western blotting

Western blot analyses revealed a dose-related induction of both P450 1A1 and P450 1A2 apoproteins in liver microsomes from I3C-treated rats after two days of exposure (Fig. 3), whereas in the small intestine only the P450 1A1 apoprotein was slightly induced. Immunostaining with a monoclonal antibody directed towards P450 2B1/2 revealed a dose-related induction of P450 2B1/2 apoprotein levels in the liver (Fig. 4).

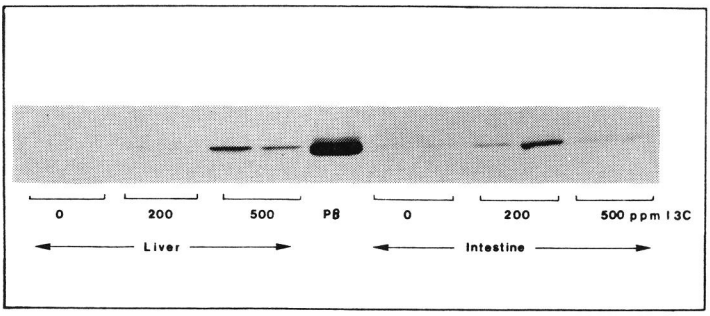

Fig. 4. SDS-PAGE-immunodetection of P450 2B1/2 apoproteins in liver and small intestinal microsomes prepared from rats treated with 0, 200 and 500 mg/kg diet indole-3-carbinol for two days. Liver microsomes prepared from a rat with phenobarbital (PB) were used as a positive control. Immunoblots were quantified with laser densitometry and results of two individual rats are given in arbitrary optical density (O.D.) units. P450 1B1 (lower band): liver 0: < 5 O.D.; 200: 12/24 O.D.; 500: 190/107 O.D.; PB: 604 O.D.; small intestine 0: < 5 O.D.; 200: 35/157 O.D.; 500: 38/35 O.D.

Table 5. The effects of dietary exposure to indole-3-carbinol on glutathione S-transferase, glucuronyl transferase and DT-diaphorase in rat liver.

Day	I3C mg/kg	GST (µmol/min/ mg protein)	GT1 (nmol/min/ mg protein)	GT2	DTD (nmol/min/ mg protein)
2	0	1.8 ± 0.2	59 ± 5	22 ± 2	141 ± 18
	200	1.9 ± 0.2	58 ± 14	23 ± 5	181 ± 33
	500	2.1 ± 0.1*	74 ± 11	25 ± 3	306 ± 149
7	0	1.9 ± 0.2	45 ± 9	18 ± 3	164 ± 75
	200	2.2 ± 0.3	67 ± 17*	22 ± 4	165 ± 39
	500	2.5 ± 0.3*	74 ± 12*	23 ± 3	510 ± 418
14	0	2.0 ± 0.5	33 ± 10	15 ± 6	79 ± 14
	200	2.5 ± 0.4	48 ± 12	19 ± 3	159 ± 80
	500	2.7 ± 0.3*	69 ± 10*	22 ± 3	219 ± 80*
28	0	2.1 ± 0.2	33 ± 10	16 ± 5	
	200	2.5 ± 0.2	52 ± 13*	19 ± 4	ND
	500	2.7 ± 0.3*	60 ± 11*	20 ± 41	

Values are means ± SD for five determinations. Those marked with asterisks differ significantly (ANOVA + Dunnetts' test) from the corresponding control value (* $P \leq 0.05$). ND = not determined.

In the small intestine from rats exposed to 200 mg/kg diet the P450 2B1/2 apoprotein levels seem to be even more induced than in the intestine of rats treated with the highest dose of I3C.In the liver of I3C-fed animals (500 mg/kg diet) P450 3A apoprotein was slightly enhanced (control 120 arbitrary optical density units (O.D.); 200 mg I3C/kg diet: 151 O.D.; 500 mg I3C/kg diet: 176 O.D.). The monoclonal antibody towards P450 3A cross-reacted with two other proteins in the small intestine, both with higher molecular weights than the P450 3A apoprotein in liver microsomes ofdexamethasone-treated rats (results not shown). Exposure of I3C slightly enhanced P450 2E, but not P450 4A apoprotein levels in the liver, whereas these apoproteins could not be detected in microsomes prepared from the small intestine (results not shown).

Phase II enzymes in liver and small intestine

The effects of dietary I3C on GST, GT1, GT2 and DTD activities in the liver are given in Table 5. Hepatic GST activity was increased from 2 days onwards in animals exposed to 500 mg I3C/kg diet (*ca.* 1.3-fold). Both the 200 and the 500 mg/kg-diets increased the GT1 activity in the liver from 7 days onwards (range: 1.5-fold to 2.1-fold). No differences were observed in hepatic GT2 activities. DTD activities were enhanced in the liver from animals fed 500 mg I3C/kg diet, but only significantly different from the control group after 14 days of exposure. Intestinal GST and DTD activities were significantly increased (to *ca.* 1.5-fold) after 14 and 28 days in rats exposed to the highest dose I3C (Table 4).

Table 6: The effects of several indole compounds on P450-dependent and GST activities in primary cultures of rat hepatocytes.

treatment	EROD	6β-OHT	7α-OHT	16α-OHT	2α-OHT	GST
	percentage of control (%)					
control (DMSO)	100	100	100	100	100	100
indole-3-carbinol	182	95	100	81	76	163
indole-3-carboxaldehyde	122	86	102	84	89	152
indole-3-acetonitrile	421	141	135	58	48	140
3,3'-diindolylmethane	2690	127	171	62	58	129

Data from test compounds comprise the results of two individual experiments. In each experiment hepatocytes of minimal six culture dishes were pooled for preparation of microsomes and cytosol. Control values are composed of data from six individual experiments: 100%-values: EROD = 12.3 ± 5.1; 6ß-OHT = 16.4 ± 5.6; 7α-OHT = 14.2 ± 5.9; 16α-OHT = 204.1 ± 59.1; 2α-OHT = 108.5 ± 8.7 pmol/min/mg protein; GST = 808.5 ± 178.1 nmol/min/mg protein.

Cytochrome P450-dependent and GST activities in cultured hepatocytes

Treatment of cultured hepatocytes with I3C and its oxidative metabolite I3CA resulted in minor changes in P450-dependent hydroxylation of testosterone and EROD activity (Table 6). In contrast, in cells exposed to I3A and DIM enhanced EROD activities were measured (4- and 27-fold, respectively), whereas the 16α- and 2α-hydroxylation of testosterone were decreased to *ca* 55% of the control group. The GST activity was enhanced in cells treated with all four indole-compounds, with the highest induction measured after exposure to I3C (1.6-fold).

Discussion

The data presented in this study indicate changes in several P450 enzymes in liver and small intestine in rats exposed to I3C. Both P450 1A1, 1A2, 2B1 and - to a lesser extent - P450 3A apoproteins were induced in the liver already after two days of exposure. The induction of P450 apoproteins was associated with enhanced EROD and PROD (marker activities for hepatic P450 1A and 2B, respectively [20]) and 16β-hydroxylation of testosterone (predominantly catalysed by P450 2B1 [25]). The slightly enhanced P450 3A levels in the liver are mirrored by enhancement of the testosterone 6β-hydroxylation [13]. Since no differences were measured in the formation of 7α-OHT we conclude that I3C exposure does not affect P450 2A, the enzyme which predominantly catalyses testosterone at the 7α-site [26].

In contrast to the variety of effects on P450 enzymes in the liver, the strong enhancement of PROD, as well as of 16α- and 16β-hydroxylation of testosterone in the small intestine is associated with solely one P450, namely P450 2B1 [25]. The higher EROD activity after I3C exposure in the small intestine could also be due to an aspecific catalytic activity of P450 2B1 because only relatively low levels of P450 1A1 apoprotein could be detected. Bradfield & Bjeldanes reported a high induction of AHH activity in the intestine of rats after treatment with 500 mg I3C/kg diet for 10 days [8]. However, no immunochemical data were given in this study. Feeding rats Brussels sprouts for only two days enhanced the EROD activity and P450 2B1 apoprotein levels in the small intestine, whereas no induction of P450 1A1 apoprotein could be detected [12]. Similar discrepancies in substrate-specificity for different P450 forms are more and more revealed. Nakajima *et al* concluded that in untreated rats the P450 2C11 and 2C6 forms are responsible for the EROD activity in the liver and not cytochrome P450 1A1 [27]. Sesardic *et al* reported a tissue-dependent difference for the phenacetin O-deethylation, which is specifically catalysed by P450 1A2 in the liver, whereas in extrahepatic tissues from 3-methylcholanthrene treated animals P450 1A1 (in absence of P450 1A2) is the predominant form to catalyses this reaction [28]. In agreement with the induction of P450 2B in the small intestine, Otte *et al* reported a phenobarbital-like and not a β-naphthoflavone-like induction of the *in vivo* metabolism of antipyrine and metronidazole after dietary exposure of rats to myrosinase treated glucosinolates [29]. Since exposure to cruciferous vegetables and/or I3C are associated with a decrease in

carcinogenesis, the possible higher bioactivation of xenobiotics due to induction of P450s in the small intestine is of minor importance. In this regard, we propose that a concomitant increase in first-pass elimination of orally dosed carcinogens in the small intestine could result in a reduced exposure at the target site for a wide range of chemically different carcinogens.

In comparison with the effects of cruciferous vegetables I3C alone at dietary levels up to 500 mg/kg diet had only minor effects on phase II enzymes [8,9]. Other glucosinolate breakdown products, such as goitrin and isothiocyanate are shown to be effective inducers of GST, DTD and epoxide hydrolase [18,30]. In our study the highest level of I3C enhanced hepatic and intestinal GST, too, which is in contrast to data reported by Bradfield & Bjeldanes [8]. Furthermore, a pronounced induction of GT1 activity in the liver was observed, which was even higher than the induction of GT1 by dietary exposure to 20% Brussels sprouts [12], a dose which corresponds to *ca* 200 mg/kg I3C [31]. To our knowledge, this is the first study to describe GT1 induction in rat liver after exposure to I3C *in vivo*, a phenomenon which could be of importance in the elimination of carcinogens.

After 28 days exposure to 500 mg/kg I3C the hepatic EROD and PROD activities decreased and no changes could be detected in hydroxylation of testosterone, whereas in the small intestine the effects seem to increase with time. A possible explanation could be a time-related enhancement of the biotransformation of these indole-compounds in the small intestine thereby reducing the actual exposure of the liver.

However, little is known about the metabolic fate *in vivo* of I3C and its condensation products in rodents [32]. Bradfield & Bjeldanes were the first to report that I3C administered i.p. did not induce the EROD activity in the liver in contrast to I3C administered orally [33]. However, when I3C was treated with 0.05 M hydrochloric acid (pH 1.5) and the reaction mixture was given i.p. a similar EROD induction was observed as measured after orally administered I3C [33]. Also under more physiological gastric pH levels (pH 2-5) I3C rapidly condensates to several indole-oligomers, among them DIM [34]. Preliminary studies revealed that treatment of rat hepatocytes with either the purified indole-oligomers or a mixture of acid-treated I3C both markedly enhanced the EROD activities [35]. These data correlate well with the fact that I3C *in vitro* had no effect on P450-dependent activities, whereas exposure of cells to DIM resulted in a strongly enhanced EROD activity and a moderate enhancement of 6β-OHT. The latter two changes were also observed after exposure to I3C *in vivo*. Furthermore, DIM reduced the 2α-hydroxylation of testosterone (P450 2C11) *in vitro*. A similar reduction of this P450 catalytic activity was observed in the liver from rats exposed to Brussels sprouts [12], but not after dietary exposure to I3C. Whether other derivatives of I3C induce 2α-hydroxylation of testosterone or other compounds in Brussels sprouts (no I3C derivatives) are responsible for the reduction of this activity remains to be investigated.

Chemical and enzymatic (myrosinase) breakdown of glucobrassicin at lower pH (pH 3-4) results in the formation of I3A and subsequently indole-3-acetic acid [1]. In the results presented here, I3A appeared to be a moderate inducer of EROD, 6β-OHT and 7α-OHT *in vitro*. Similar to DIM, a decrease of 2α-OHT was also measured after I3A treatment *in vitro*. However, it should be noted that a relatively high dose of I3A was administered to cells in comparison with the other indole compounds. I3CA, a major

oxidative metabolite of both I3A and I3C (Van Bladeren, unpublished results), did not markedly affect the P450-dependent activities *in vitro*.

Since I3C does not induce P450-dependent activities in hepatocyte cultures, while DIM is very effective, it is concluded - in agreement with Bradfield & Bjeldanes [33] - that I3C itself is not the active principal for P450 induction after consumption of I3C or Brussels sprouts. Moreover, the conversion of I3C to acid-condensation products is thought to be essential in order to establish the inductive effects [34]. Dietary exposure to I3C *in vivo* results in the induction of a broad spectrum of P450 enzymes in the liver, while in the intestinal mucosa cells predominantly P450 2B1 is affected. These changes on biotransformation activities are comparable to the effects observed after dietary exposure to Brussels sprouts. However, the acid-condensation products of I3C and/or I3A are only one group of compounds among many others in Brussels sprouts known to affect biotransformation enzymes.

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CHAPTER 6

STRUCTURE ELUCIDATION OF ACID REACTION PRODUCTS OF INDOLE-3-CARBINOL

Detection in vivo and enzyme induction in vitro

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Abstract

The potency of indole-3-carbinol (I3C) to form condensation products under acidic aqueous conditions was studied. Next to identifying a known dimer, 3,3'-diindolylmethane (DIM), we elucidated the structures of two trimers also found in acid reaction mixtures: 5,6,11,12,17,18-hexahydrocyclonona [1,2-*b*:4,5-*b*': 7,8-*b*"] tri-indole (CTI), and 2,3-bis [3-indolylmethyl] indole (BII). The formation of these indole oligomers was shown to be pH dependent. The highest amounts of DIM and BII were formed in aqueous solutions having a pH value ranging from 4 to 5. No CTI could be detected at pH values above 4.5. In rats that received an oral dose of I3C we could detect DIM and BII in gastric contents, stomach tissue, small intestine and liver. No CTI could be detected *in vivo* after oral exposure to I3C. In *in vitro* experiments, using rat hepatocytes, the cytochrome P450IA1 apoprotein level, 7-ethoxyresorufin O-deethylation activity (EROD) and DT-diaphorase activity (DTD) were markedly enhanced by DIM, CTI as well as BII.

Chem-Biol Interact (in press)

Introduction

Cruciferous vegetables such as cabbage, cauliflower and Brussels sprouts contain a variety of compounds that modulate the carcinogenicity of several chemicals [1-3]. One of these compounds is indole-3-carbinol (I3C)[3]. I3C has been reported to inhibit tumorigenesis in rodents exposed to polycyclic aromatic hydrocarbons [4,5]. However, enhancement of chemically induced carcinogenesis by I3C has also been reported [6]. It has been postulated that I3C affects chemical carcinogenesis by modulation of Phase I and Phase II biotransformation activities [6-9].

The findings of Bradfield and Bjeldanes [12] indicate that I3C mediates enzyme induction via a series of acid condensation products generated upon introduction of I3C into the acidic environment of the stomach. Oral intubation of rats with I3C led to a significant induction of hepatic 7-ethoxyresorufin O-deethylation activity (EROD). When administered i.p., I3C did not enhance hepatic EROD. An I3C acid reaction mixture, generated upon treatment of I3C with an acidic aqueous solution, enhanced EROD both after i.p. or p.o. dosing. These results were confirmed by experiments in our laboratory: the addition of an I3C acid reaction mixture to cultured hepatocytes resulted in a markedly enhanced EROD activity in these cells, whereas I3C alone did not have such an effect [13].

One of the acid condensation products has been identified as 3,3'-diindolylmethane (DIM) [10,12,14]. It has been suggested that next to DIM a series of linear and cyclic methyleneindole trimers and tetramers are formed [10,12,15,16]. No comprehensive study to the exact structure of these products has been described, however.

Limited data are available on the metabolic fate of I3C *in vivo*. Dashwood *et al* [14] studied the *in vivo* disposition of I3C in the rainbow trout. Following the administration of [5-^{3}H]I3C, radiolabeled species other than the parent compound could be recovered from bile and liver. One of these compounds was identified as DIM. No such studies have been described in mammals.

In the present study we investigated the formation, structure and some biological aspects of a number of acid condensation products of I3C. We report upon the effects of pH on the formation of these condensation products. Analysis and purification of condensation products was performed using HPLC. Mass-spectral and NMR studies were undertaken to elucidate the structure of products formed upon the acid condensation of I3C. The *in vivo* formation of condensation products was studied in rats having received an oral dose of I3C. An *in vitro* experiment, using cultured rat hepatocytes, was performed to examine whether the purified oligomers can individually enhance P450 1A1/2 apoprotein levels, 7-ethoxyresorufin O-deethylation activity (EROD) and DT-diaphorase (NAD(P)H:quinone oxidoreductase; EC 1.6.99.2; DTD) activity.

Materials and methods

Chemicals

Indole-3-carbinol and Williams' medium E were obtained from Sigma Chemical Co., St Louis, MO, U.S.A.. Newborn calf serum was purchased from Gibco Europe, Breda, The Netherlands. All other chemicals were of analytical or HPLC grade. 3,3'-diindolylmethane (DIM) was a kind gift from Ir J. Vuik (TNO-Toxicology and Nutrition Institute, Zeist, The Netherlands). A monoclonal antibody towards P450 1A1/2 was a gift from Dr P.J. Kremers, Université de Liège, Belgium. The secondary antibody was supplied by Dakopatts a/s, Glostrup, Denmark.

Condensation of I3C

An acid reaction mixture was prepared as described by Bradfield and Bjeldanes [12]. Fifty mg I3C, dissolved in 200 µl DMSO, was added slowly to 50 ml 0.05 M HCl (pH 1.5). After 4-60 min in a shaking water bath at room temperature a sample was taken, diluted 1:1 with acetonitrile and injected into the HPLC.

To investigate the effects of pH on I3C condensation, the pH value of aquadest was adjusted using HCl, resulting in a series of aqueous solutions with pH values ranging from 1.3 to 5.4. The addition of I3C and sample preparation were executed as described above.

HPLC analysis of acid condensation products

Analysis of acid condensation products were performed on an HPLC system equipped with a fraction collector (LKB, Bromma, Sweden). Separations were accomplished using a Lichrosorb 5-RP-18 (250 x 4.6 mm, 1 x i.d.) column preceded by a 10 mm RP guard column (Chrompack, Middelburg, The Netherlands), maintained at ambient temperature. I3C and its oligomers were eluted using a linear gradient from 20% acetonitrile at 0 min to 85% acetonitrile at 30 min, followed by 85% acetonitrile from 30 to 60 min. Flow rate was kept at 1.0 ml/min. Column effluents were monitored at 280 nm. Product formation was quantified by comparing peak areas to those of standards.

Preparative HPLC of acid condensation products

I3C, 1 g dissolved in 4 ml DMSO, was added slowly to 1 l 0.05 M HCl (pH 1.5). The solution was stirred for 60 min and the resulting precipitate was removed by filtration. The residue, comprising a mixture of I3C oligomers was dried at room temperature. The oligomers were dissolved in DMSO at a concentration of 100 mg/ml, of which 100 µl were repeatedly injected into the HPLC. A steel column (600 x 8.0 mm, 1 x i.d.) packed with RP-C18, 40 µM particle diameter (J.T. Baker Chemical Co., Phillipsburg, U.S.A.) was used for separations. The oligomers were eluted using 62% acetonitrile from 0 to 40 min followed by 100% acetonitrile from 40 to 75 min, at a flow rate of 2.5 ml/min. Acetonitrile was removed from collected samples using a rotary vacuum evaporator. Oligomers were extracted from the resulting aqueous phase with dichloromethane. Removal of the dichloromethane *in vacuo* yielded 30 - 50 mg purified products. The first 3 major components as shown in Fig. 1A were purified by this method.

HPLC-MS analysis of acid condensation products

HPLC-MS analyses were performed on a Finnigan MAT TSQ-70 mass spectrometer equipped with a Finnigan MAT thermospray interface (Finnigan MAT, San José, CA, USA). The HPLC system used was as described above. Scans were made from *m/z* 100 to 800 in 5 s/scan; the source temperature was set at 230°C, the vaporizer temperature at 110°C and the repeller potential at 50 V. Discharge-on ionisation was applied (1 kV).

NMR analysis of purified products

The NMR-spectra were recorded on a 400 MHz Varian Unity 400 spectrometer (Varian, CA, USA), using standard pulse programs. The samples were prepared by dissolving 20-30 mg of the purified fractions in 0.6 ml DMSO-d6 to which tetramethyl silane was added as an internal reference. A 5 mm switchable probe was used. The spectra were recorded at 30°C.

Animals

Male Wistar rats ((Ico:WU), Iffa-Credo, Someren, The Netherlands), weighing 200 - 350 g, were fed *ad libitum* a semisynthetic diet consisting of 13% (w/w) casein, 5% cellulose, 5% corn-oil, 30% wheat starch, 13% soy-protein isolate, 30% sucrose, 3.5% mineral mix, 0.3% vitamin ADEK preparation and 0.2% vitamin B mixture. The animals had free access to drinking water.

Formation of acid condensation products in vivo

Rats were given I3C, suspended in water (pH 7.3), by gavage at a dose of 200 μmol/kg bodyweight. Control animals received water only. After 1 h the rats were anaesthetized using pentobarbital-sodium (Nembutal, Algin BV, Maassluis, The Netherlands) at a dose of 90 mg/kg bodyweight. Livers were perfused with saline via the portal vein to remove the blood. The stomachs were excised and the contents collected by flushing the tissue with 5 ml PBS. PBS was used to further clean the stomach tissue. The proximal 20 cm of the small intestines were excised and perfused with 10 ml PBS. Tissues and stomach contents were stored at -20°C. Condensation products were extracted from tissues and gastric contents with 2% iso-amylalcohol in dichloromethane. Tissues were minced with scissors and homogenized directly in the extraction solvent using an ultra-turrax. The organic phase was separated by centrifugation (15 min at 1000 *g*) and evaporated under a stream of N_2 at room temperature. The residue was dissolved in 1 ml acetonitrile, diluted 1:1 with 0.1 M Na-phosphate buffer (pH 7.8) and injected into the HPLC.

Cell isolation and culture

Isolation and culture of rat hepatocytes was performed as described previously [17]. After a total preincubation time of 24 h, cultures were treated for 48 h with indole oligomers, dissolved in DMSO, or with DMSO alone. The final concentration of DMSO was 0.1%.

Preparation of microsomes and cytosol

Microsomes and cytosol were prepared as described previously [17]. Protein contents were determined according to Rutten *et al* [18].

7-Ethoxyresorufin O-deethylation (EROD) and DT-Diaphorase (DTD)

Fluorometric determinations of EROD activities were performed directly in intact hepatocyte monolayers as described by Wortelboer *et al* [17]. DTD was assayed fluorometrically by the reduction of resorufin, basically as described by Spencer and Rifkind [19]. Briefly, reaction mixtures contained 0.05 M Tris-HCl buffer (pH 7.5), 15 μM resorufin (dissolved in DMSO and diluted 250-fold in Tris-HCl), 400 μM NADPH and 50-100 μg cytosolic protein in a final volume of 1.0 ml. The decrease in fluorescence of resorufin was monitored at 25°C for at least 3 min using a Kontron SFM 25 fluorescence spectrophotometer (Kontron, Zürich, Switzerland).

Gel electrophoresis and immunoblotting

Microsomal proteins were separated on a Biorad mini Protean II cell by sodium dodecyl sulphate gel electrophoresis (SDS-PAGE), essentially as described by Laemmli [20]. Following electrophoresis, the proteins were transferred to polyvinylidene difluoride sheets as described by Towbin *et al* [21]. P450 1A1/2 apoproteins were detected using a monoclonal antibody directed towards these isoenzymes.

Results

Condensation of I3C

Fig. 1A shows an elution profile of I3C condensation products obtained by incubating I3C in 0.05 M HCl (pH 1.5) for 60 min. Under these conditions at least 15 products were formed. The first major peak in the chromatogram co-eluted with DIM. The formation of I3C condensation products could not be influenced by saturating reaction mixtures with either O_2, N_2 or CO_2. The condensation of I3C proceeded rapidly and irreversibly; the formation of condensation products was complete in 4 min and no changes were observed to occur thereafter. Readjustment of the pH value of the reaction mixture to 7-8, using NaOH, did not result in the loss of I3C condensation products (results not shown).

HPLC-MS and NMR analysis of acid condensation products

A mixture of oligomers prepared by acid treatment of I3C was subjected to HPLC-MS analysis. Major peaks as shown in Fig. 1A exhibited parent ion peaks at *m/z* 247, 388, 376, 372, 372, 505 and 634. The identity of the first peak, DIM, is confirmed by *m/z* 247.

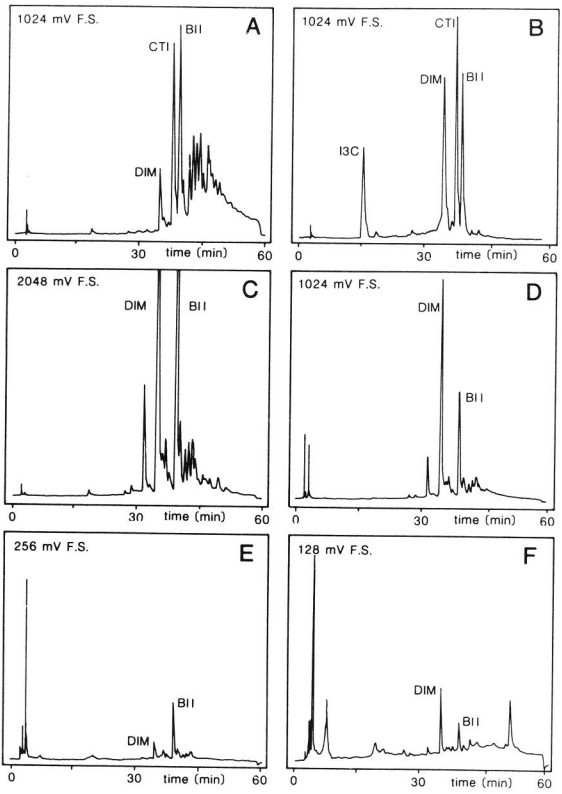

Fig. 1. HPLC elution profiles of indole derivatives. (A), I3C in 0.05 M HCl; B, purified components, *ca.* 4 nmol of I3C, DIM, CTI and BII. After oral administration of I3C to rats, indole oligomers could be extracted from gastric contents and tissues: (C), gastric contents; (D), stomach tissue; (E), small intestine; (F), liver. The peak having an R_t value of 32.5 min in Figs. C and D is thought to represent an unknown indole compound. Non-annotated major peaks in Fig. F are of endogenous origin. No peaks interfering with the detection of the indole oligomers were encountered in HPLC analyses of gastric contents and tissues from control rats. The results shown are from one typical experiment out of four. Note: recordings were made at different attenuations as displayed in the top-left corner of each figure.

NMR-analysis was performed to elucidate the structure of products formed upon the acid condensation of I3C. Final results are given in Fig. 2. The ^{1}H and ^{13}C NMR spectra of the first major peak as shown in Fig. 1A were identical to those of an authentic sample of 3,3'-diindolylmethane (DIM). The δH assignments were: 10.71 (H-1, J 2, 3 Hz), 7.12 (H-2, J 2.3 and 0.9 Hz), 7.52 (H-4, J 7.6 and 1.2 Hz), 6.92 (H-5, J 7.6, 7.2 and 1.0 Hz), 7.03 (H-6, J 8.1, 7.2 and 1.2 Hz), 7.32 (H-7, J 8.1 and 1.0 Hz),

4.13 ((-CH_2-), J 0.9 Hz). The δC assignments were: 20.80, 111.18, 114.11, 117.91, 118.53, 120.62, 122.63, 127.11 and 136.30.The ^1H and ^{13}C NMR spectra of the second major peak in Fig. 1A showed the same signals as those which have already been reported for 5,6,11,12,17,18-hexahydrocyclonona[1,2-b;4.5-b';7,8-b"]tri-indole (CTI) [22]. Minor differences in observed chemical shifts can be explained by differences in temperature or solvent. The δH assignments were: 3.94 (1H, J 15 Hz, CH_2), 4.72 (1H, J 15 Hz, CH_2), 6.8-7.0 (2H, ArH), 7.19 (1H, ArH), 7.97 (1H, ArH), 10.73 (1H, NH). The δC assignments were: 22.03, 108.73, 111.20, 118.22, 119.32, 121.14, 129.46, 135.46 and 135.96.

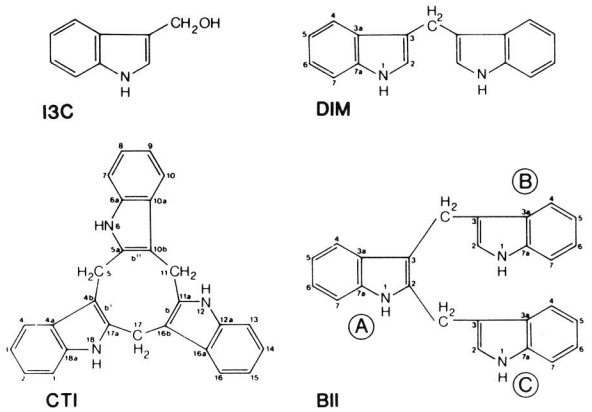

Fig. 2. Structures of indole derivatives. I3C, indole-3-carbinol; DIM, 3,3'-diindolylmethane; CTI, 5,6,11,12,17,18-hexahydrocyclonona [1,2-*b*:4,5-*b*':7,8-*b*":] tri-indole; BII, 2,3-bis [3-indolylmethyl] indole.

The ^1H and ^{13}C NMR spectra of the third major peak in Fig. 1A showed the presence of a 2,3 disubstituted indole group, (A), with two indolyl methyl groups, (B) and (C) (Fig. 2D). This structure has been reported previously, though the methylene attachment points were left undetermined [22]. This uncertainty has now been removed by means of HC-correlation spectroscopy; the structure was determined to be 2,3-bis (3-indolylmethyl) indole (BII). The δH assignments were: A; 7.36 (H-4, J 7.8/1.2), 6.83 (H-5, J 7.8/1.0), 6.92 (H-6, J 1.2), 7.22 (H-7, J 1.0), 10.56 (H-1). B; 7.47 (H-4, J 8.0/1.2), 6.86 (H-5, J 8.0/7.1/1.2), 7.02 (H-6, J 7.1/8.1/1.2), 7.30 (H-7, J 8.1/1.2), 10.64 (H-1), 4.19 (CH_2). C; 7.39 (H-4, J 7.8/1.0), 6.86 (H-5, J 7.8/7.1/1.0), 7.03 (H-6, J 7.1/8.1/1.0), 7.33 (H-7, J 8.1/1.0), 10.81 (H-1), 4.23 (CH_2). The δC assignments were: 136.30, 136.23, 135.33, 134.69 (C-2, A and C-7a, BC), 128.32, 127.09, 126.86 (C-3a,

ABC), 123.16 (C-2, C), 122.61 (C-2, B), 120.81 (C-6,C), 120.59 (C-6, B), 119.72 (C-6, A), 118.48 (C-4, B), 118.38 (C-4, C), 118.10 (C-4, A), 118.01 (C-5, B), 117.88 (C-5, C), 117.74 (C-5, A), 114.56, 112.07, 109.12 (C-3, ABC), 111.19 (C-7, C), 111.13 (C-7, B), 110.50 (C-7, A), 21.98 (CH_2, C), 1971 (CH_2, B). Due to the complexity of the ^1H spectrum it was impossible to determine every coupling constant. However, coupling between protons has been verified by means of COSY (Correlation Spectroscopy)-spectra, in which the correlation chains clearly marked the resonances of the protons belonging to the moieties A, B and C. The COSY-spectrum also showed a correlation between the amide proton of fragment A and the CH_2 group of fragment C, whereas no correlation has been found between this amide proton and the CH_2-group of fragment B. This leads to the conclusion that fragment C is attached to the 2-position of fragment A. Knowing the proton shifts, the shifts of the proton-bearing carbons in the molecule were easily determined by an HC-correlation spectrum. The identity of the carbon atoms (C-2, C-3, C-3a, C-7a) in the moieties A, B and C, respectively, were not determined; appropriate chemical shifts may be exchanged without affecting the proof of the structure of this compound.

pH dependence of I3C condensation

The formation of oligomers was strongly pH-dependent (Fig. 3). At a pH value below 3 the oligomers DIM, CTI and BII were formed in approximately equal amounts. Incubation of I3C at a pH value above 3 resulted in the formation of larger amounts of DIM and BII and smaller amounts of CTI. At pH values of 4.5 and higher the formation of BII declined. No CTI could be detected to be formed at pH values above 4.5.

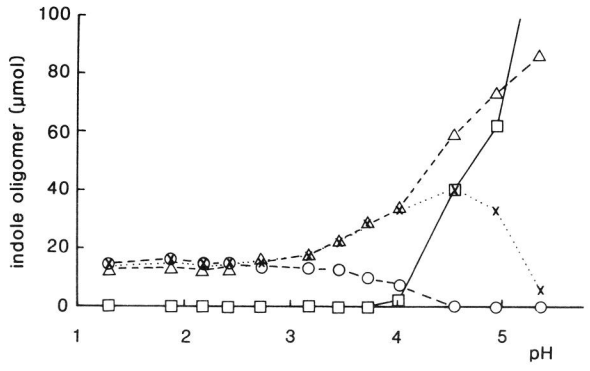

Fig. 3. The pH dependence of the formation of DIM, CTI and BII. I3C (340 µmol) was added to aqueous solutions with pH values ranging from 1.5 to 5.5. After 60 min indole oligomer contents were determined by HPLC. (□), I3C; (△), DIM; (○), CTI; (**x**), BII.

Formation of acid condensation products in vivo

The HPLC profile of indole derivatives extracted from the gastric contents of rats treated orally with I3C (Fig. 1C) was similar to the profile obtained from the acid reaction mixture of I3C (Fig. 1A). No I3C could be detected. A relative abundance of DIM and BII, compared to the other polymers, could be observed in the gastric contents. In a typical experiment, the amounts of DIM and BII recovered from the stomach contents comprise 3.5% and 4.4% (w/w) respectively of the administrated dose of I3C. No peak having an R_t value corresponding to those of CTI could be identified, while an additional major peak with a R_t value of 32.5 min was detected in these samples. In extracts from stomach tissue, small intestine and liver a pattern of I3C oligomers similar to that found in the stomach contents was detected (Fig. 1D-1F). DIM and BII could be identified in all tissues, CTI in none of the tissues.

Enzyme induction in cultured hepatocytes

Exposure of cultured hepatocytes to indole oligomers resulted in a high increase in EROD activity. The EROD activities were enhanced 17.3-, 11.8- and 19.5-fold by DIM, CTI and BII respectively (Fig. 4). Corresponding to this, high levels of P450 1A1 apoprotein were revealed in microsomes prepared from these hepatocytes (Fig. 5).

DT-diaphorase activities towards resorufin were enhanced 2.8-, 2.3- and 3.6-fold by DIM, CTI and BII respectively (Fig. 6).

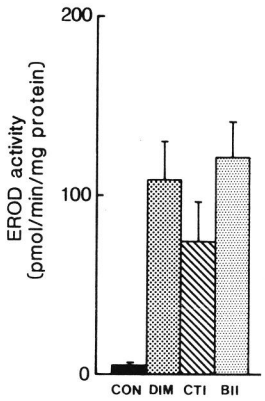

Fig. 4. 7-Ethoxyresorufin O-deethylation activity (EROD) in rat hepatocytes cultured for 48 h in media supplemented with 25 µM indole oligomers.

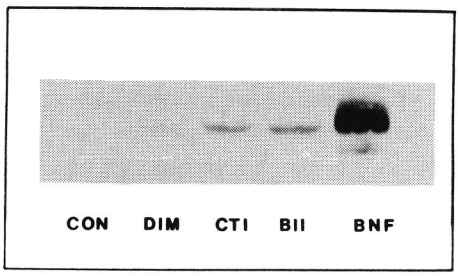

Fig. 5. SDS-PAGE-immunodetection of P450 1A1/2 apoproteins in microsomes prepared from rat hepatocytes cultured for 48 h in media supplemented with 25 µM indole oligomers. Liver microsomes prepared from a rat treated with ß-naphthoflavone (BNF) were used as a positive control.

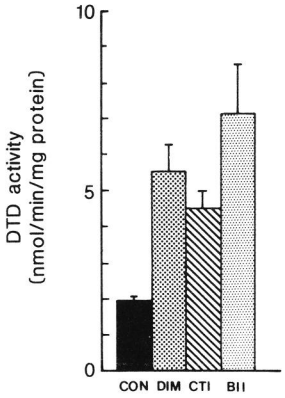

Fig. 6. DT-diaphorase activity (DTD) in rat hepatocytes cultured for 48 h in media supplemented with 25 μM indole oligomers.

Discussion

To our knowledge, this paper is the first one to present the chemical structures of I3C acid condensation products other than DIM. These structures were characterized as the methyleneindole polymers CTI and BII. The structures of the trimers CTI and BII have been described before, though as endogenous compounds in the sponge *Pachymatisma johnstoni* [22]. The formation of methylene indole polymers in I3C acid condensation reactions has been suggested previously [12,15,16]. Fong *et al* [10] have presented evidence that next to DIM two oligomers exhibiting parent ion peaks at *m/z* 386 and 382 are formed in high amounts in I3C acid reaction mixtures. Though the *m/z* value of 386 corresponds well with the molecular weight of CTI, we did not detect parent ion peaks at *m/z* 382 in our studies. Interestingly, *m/z* 505 and 634, as found in our studies, can both be explained by adding the molecular weight of one or more methyleneindole groups (130) to the molecular weight of BII (375). This suggests that further polymerisation proceeds by the addition of methyleneindole groups to the indolylmethyl groups of BII.

The pattern of acid condensation products in the gastric contents of rats after oral intubation of I3C strongly resembled the oligomer pattern obtained after addition of I3C to an aqueous solution with a pH value of 4.5 to 5: in both cases the formation of DIM and BII could be shown to occur readily whereas no CTI could be detected (Figs. 1C and 3). The fact that we could not detect CTI after *in vivo* exposure to I3C can be explained by the pH value of the gastric contents of well-fed rats. The gastric contents of rats fed our diet had pH values ranging from 4.5 to 5.5 (n=3). The pH of gastric juice in an empty stomach may be less than 2, when food enters the stomach the buffers (primarily protein) contained in the food will neutralize the acid [23]. Furthermore, not only the relatively high pH of the gastric contents will inhibit the formation of CTI. The pH value of a 30% (w/v) emulsion of rat diet in 0.05 M HCl is approximately 5.0. When the pH of this emulsion is lowered to 1.5, using HCl, the addition of I3C still does not result in the detection of CTI, though DIM and BII are readily formed (results not shown). Together these results strongly suggest that CTI will not be formed in the stomach. These

findings implicate that an I3C acid reaction mixture, prepared to study I3C-mediated actions, should preferentially be made at a pH value of 4.5, resulting in a mixture of methylene indole oligomers more representative of the mixture generated *in vivo*. The presence of DIM and BII in stomach tissue and small intestine suggests that these compounds are absorbed from the gastrointestinal system. This is confirmed by the presence of DIM and BII in the liver (Fig. 1F).

The potency of orally administered I3C to enhance hepatic EROD activity (P450 1A1) has been well described [12,24]. Bradfield and Bjeldanes [12] demonstrated that this effect could be partly attributed to the action of DIM, formed upon the introduction of I3C into the acidic environment of the stomach. We have shown that other I3C acid condensation products have a similar EROD-inducing capacity (Fig.4). These results are confirmed by the *de novo* synthesis of P450 1A1 apoprotein (Fig.5) in hepatocyte cultures in response to these indole oligomers. An increase in hepatic DTD activity in response to orally administered I3C has been reported by Salbe and Bjeldanes [8]. The increase in DTD activity in hepatocytes exposed to respectively DIM, CTI and BII (Fig.6) indicates that DIM and BII could be responsible for the hepatic DTD induction found after exposure to I3C *in vivo*.

In the rat, DTD activity is coordinately enhanced with cytochrome P450 1A mediated activities by *Ah* receptor ligands like 3-methylcholanthrene, benzo[*a*]pyrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [25,26]. Prochaska and Talalay [27] have demonstrated, using a murine hepatoma cell line, that bifunctional inducers which induce both P450 1A and DTD require *Ah* receptor binding for their action. Our findings that both EROD and DTD are induced in hepatocytes exposed to indole oligomers suggest that these substances act by means of *Ah* receptor binding. However, this hypothesis is not in accordance with the work of Gillner *et al* [28] who reported that DIM does not interact significantly with the *Ah* receptor. No such data are available for CTI and BII. It is plausible that DIM, CTI and BII are metabolized to high affinity receptor ligands, such as indolo[3,2-*b*]carbazole, though no such metabolism studies have been described yet.

In conclusion, this study reports the identification of two indole trimers formed in I3C acid reaction mixtures. Both trimers were shown to be potent enhancers of specific biotransformation activities *in vitro*. Identification of BII in the gastro-intestinal tract of I3C treated rats suggests that BII is a major indole condensation product active *in vivo*.

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CHAPTER 7

ACID REACTION PRODUCTS OF INDOLE-3-CARBINOL AND THEIR EFFECTS ON CYTOCHROME P450 AND PHASE II ENZYMES IN RAT AND MONKEY HEPATOCYTES

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Abstract

The effects of three acid condensation products of indole-3-carbinol (I3C), i.e. 3,3'-diindolylmethane (DIM), 5,6,11,12,17,18-hexahydro-cyclonona [1,2-*b*:4,5-*b*':7,8-*b*":] tri-indole (CTI) and 2,3-bis [3-indolylmethyl] indole (BII), on cytochrome P450 (P450) and phase II enzymes were studied in primary cultures of rat and cynomolgus monkey liver cells. In rat hepatocytes all three indole derivatives dose-relatedly induced the ethoxyresorufin O-dealkylation (EROD) activity (to 24-fold) and 7α-hydroxylation of testosterone (to 4-fold), whereas all three decreased the 16α- and 2α-testosterone hydroxylation (DIM to 60%, CTI and BII to a mere 5% of the control cells). Treatment of monkey hepatocytes with DIM and BII enhanced the EROD activity to 6 and 9-fold, respectively. Furthermore, BII decreased the 6β-hydroxylation of testosterone (to 60% of the untreated cultures) in monkey cells.

Phase II enzymes were also affected. In rat hepatocytes DIM, CTI and BII enhanced DT-diaphorase activity, and DIM and BII the glucuronidation of 1-naphthol. In monkey cells BII only resulted in enhanced DT-diaphorase, and no changes were observed in glucuronidation of 1-naphthol after treatment with either of DIM or BII. The indole derivatives did not affect glutathione S-transferase activity and sulphation of 1-naphthol in either rat or monkey hepatocytes. These results identify two novel acid-condensation products of I3C, CTI and BII as potent compounds in affecting biotransformation in rat as well as in monkey hepatocytes.

Biochem Pharmacol (submitted)

Introduction

The human diet contains a number of compounds that affects the carcinogenic process. Apart from carcinogens our food contains compounds which prevent tumorigenesis, among them minor non-nutrient constituents of vegetables and fruits, such as terpenes, aromatic isothiocyanates, phenols, flavones and indoles [1-3]. Indoles are hydrolysis products of indole-glucosinolates, a group of compounds in cruciferous vegetables (e.g. Brussels sprouts, cabbage and cauliflower) [4]. One of the major hydrolysis products is indole-3-carbinol (I3C). A high consumption of cruciferous vegetables has been related to a decreased risk in carcinogenesis in man [3,4], and a decreased chemically-induced tumorigenesis in laboratory animals [4,5]. In contrast, other studies indicated an enhanced promotor effect of I3C on tumorigenesis in rats [6].

Since many carcinogens are subjected to metabolic activation and inactivation, changing the biotransformation enzyme activities may be an effective means to affect the carcinogenic potential of these xenobiotics. Dietary exposure to cruciferous vegetables or I3C is known to enhance several cytochrome P450 enzymes and other drug-metabolizing enzymes [4,5,7]. Recently, we described the induction of four different P450 forms (P450 1A1, 1A2, 2B1 and 3A) in the liver of rats fed I3C for as short as two days [8]. Accordingly, the dealkylation of ethoxy- and pentoxyresorufin (EROD & PROD), and the 6β-hydroxylation of testosterone were enhanced. Longer exposure to I3C also enhanced several phase II enzymes such as glucuronyl transferase, DT-diaphorase and glutathione S-transferase [8-10].

In an earlier study, Bradfield & Bjeldanes [11] also reported induction of EROD activity in the liver of rats orally exposed to I3C. However, when I3C was given i.p. no enhancement of EROD activity could be measured. The researchers suggested that the conversion of I3C in the acid environment of the stomach was essential for its inducing effects. In a study at our own laboratory treatment of hepatocytes with I3C itself had only minor effects on biotransformation enzymes whereas the dimer 3,3'-diindolylmethane (DIM) and a complete acid-reaction mixture of I3C markedly enhanced the EROD activity [12]. Within this acid-reaction mixture, we identified the chemical structures of two trimers i.e. 5,6,11,12,17,18-hexahydrocyclonona [1,2-*b*:4,5-*b*':7,8-*b*"] tri-indole (CTI), and 2,3-bis [3-indolylmethyl] indole (BII), in addition to the already known dimer DIM [13].

All three indole derivatives induced cytochrome P450 1A1 and its associated EROD activity [14] in rat hepatocytes [13]. Induction of cytochrome P450 1A1, however, can be related with metabolically activation of pro-carcinogens to reactive intermediates, giving rise to toxicity and carcinogenicity [15]. Since dietary exposure to I3C generally decreases tumorigenesis *in vivo* we were interested to know whether the acid condensation products of I3C are capable in affecting other P450 forms and/or phase II enzymes.

Primary cultures of hepatocytes are a useful alternative in studying the changes in biotransformation activities catalysed by several P450 forms [16-18]. One of the advantages in comparison to *in vivo* studies is that only small amounts of purified compounds are needed to elucidate the inductive capacity of these compounds. Moreover, a comparison of the effects between rat and human hepatocytes can improve human risk

evaluation. Human cells, however, are not easily available. Hepatocytes from monkey liver might be a good alternative for human hepatocytes [19].

In the present study we investigated the effects of three indole-oligomers - DIM, CTI and BII - on P450 pattern and several phase II enzymes in rat hepatocytes in more detail. In comparison, the effects of I3C, DIM and the trimer BII on biotransformation activities were studied in hepatocytes derived from cynomolgus monkey (*Macaca fascicularis*).

Materials and methods

Materials

Newborn calf serum was obtained from Gibco Europe (Breda, The Netherlands). Indole-3-carbinol, Williams' E medium, androstenedione, 11β- and 16α-hydroxy-testosterone (11β- and 16α-OHT) were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). 2α-OHT was a gift from Prof D.N. Kirk (Queen Mary College, University of London). 15β-OHT was a gift from G.D. Searle and Co. (Skokie, IL, U.S.A.), 6β-, 7α- and 16β-OHT were obtained from Steraloids (Wilton, NH, U.S.A.). 3,3'-diindolyl-methane (DIM), 5,6,11,12,17,18-hexahydrocyclonona [1,2-*b*:4,5-*b*':7,8-*b*"] tri-indole (CTI), and 2,3-bis [3-indolylmethyl] indole (BII) were purified from an acid-reaction mixture of I3C as described before [13]. All other chemicals were of analytical grade. Monoclonal antibodies towards P450 1A1/2, P450 2B1/2 and P450 3A were kind gifts from Dr P.J. Kremers, Université de Liège, Belgium. Secondary antibodies were obtained from Dakopatts a/s, Glostrup, Denmark.

Animals and cell isolation

Male Wistar rats (Ico:WU, Iffa-Credo, Someren, The Netherlands), weighing 200 - 350 g, were fed *ad libitum* a semisynthetic diet as described previously [8]. The animals had free access to drinking water. Hepatocytes were isolated using a two step collagenase perfusion technique as described before [17]. Cynomolgus monkeys (*Macaca fascicularis*) were bred at the National Institute of Public Health and Environmental Protection (RIVM, Bilthoven, The Netherlands). The monkeys served as donors for kidney cells necessary for the production of the poliomyelitis vaccine. Liver cells were isolated as described by Mennes *et al.* [20].

Cell culture

The cells were plated on 6 or 9 cm tissue culture dishes (Sterilin) at a density of 4 or 8 x 10^6 cells/dish in 4 or 10 ml Williams' E medium, respectively. Media were supplemented with 3% (rats) or 5% (monkeys) newborn calf serum, 1 μM insulin, 10 μM hydrocortisone and 50 mg/l gentamycin. Additionally, media of monkey hepatocytes were supplemented with 4 mM $CaCl_2$ and 4 mM $MgCl_2$ during the first 4 hr in culture. Cells were incubated in a humidified atmosphere of air (95%) and CO_2 (5%) at 37°.

After 4 hr in culture, media were replaced. Thereafter, media were refreshed every 24 hr. After a total preincubation period of 24 hr, I3C, DIM, CTI and BII dissolved in

dimethyl sulfoxide (DMSO), were added to the culture media to give a final concentration range of 2.5 to 40 μM. An equal amount of DMSO was added to the control cultures (final DMSO concentration 0.1% v/v). Monkey hepatocytes were treated with only one concentration of either I3C, DIM or BII (25 μM).

Biochemical determinations

The leakage of lactate dehydrogenase (LDH, EC 1.1.1.27) was used as an indication for cell viability. The LDH activity was measured according to Bergmeyer *et al.* [21]. Microsomes and cytosol were prepared, and protein contents were determined both as described previously [22]. Determinations of 7-ethoxyresorufin deethylation (EROD) and hydroxylation of testosterone were performed directly in intact hepatocyte monolayers as described by Wortelboer *et al.* [22]. Glutathione S-transferase (GST) activity was measured in cytosol with 1-chloro-2,4-dinitrobenzene (5 μM) as a substrate using the spectrophotometric method of Habig *et al.* [23]. DT-diaphorase (DTD) activity was assayed fluorimetrically by the reduction of resorufin, as described previously [13].

Glucuronyl transferase (GT) and sulfotransferase (ST) activities were measured directly in intact hepatocyte monolayers. Cells were washed twice with Hanks balanced salt solution (HBSS) and incubated with 250 μM 1-naphthol in HBSS at 37° in a humidified atmosphere of air (95%) and CO_2 (5%) at 37°. After 25 and 50 min samples of one ml were taken and stored at -20°C. Media were centrifuged (5 min, 300 ***g***) and 1-naphthyl-glucuronides and 1-naphthyl-sulphates were analysed by HPLC according to Redegeld *et al* [24] with some modifications. A Chromsep C18 RP column (200 x 3 mm, 1. x i.d., Chrompack, Middelburg, The Netherlands) was used for separations. Products were eluted with a mixture of 17.5% acetonitrile in 10 mM KH_2PO_4, pH 2.5 at a flow rate of 1.0 ml/min, giving a typical elution profile as shown in Fig. 1. Column effluents were monitored fluorimetrically using an excitation wavelength of 290 nm and an emission wavelength 330 nm. Metabolites were quantified by comparing peak areas with those of authentic standards.

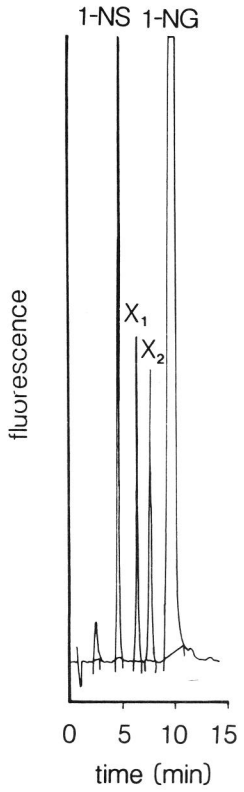

Fig. 1: HPLC elution profile of 1-naphthyl-conjugates (1-NG: glucuronide and 1-NS: sulphate) in medium from intact monolayers of control rat hepatocytes incubated with 250 μM 1-naphthol for 50 min. X1 and X2 are unidentified.

Gel electrophoresis and immunoblotting

Microsomal proteins (2 μg protein) were electrophoretically separated using a Biorad mini Protean II cell by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and subsequently immunoblotted as described [17]. P450 1A1/2, P450 2B1/2 and P450 3A apoproteins were detected using monoclonal antibodies directed towards these isoenzymes.

Statistical analysis

Results have been expressed as means ± SD, where appropriate. Statistical analysis was performed by Students' *t*-test, for unpaired samples ($P \leq 0.05$).

Results

Effects of indole derivatives on P450 enzymes in rat hepatocytes

Incubation of rat cells with the indole-derivatives up to 40 μM did not result in significant loss of cell viability as measured by LDH-leakage (results not shown). All three acid-condensation products of I3C enhanced the EROD activity dose-relatedly (Fig. 2A). At a concentration of only 3 μM and upwards a significant enhancement of EROD could be detected. At a concentration as high as 40 μM, however, the EROD enhancement due to CTI exposure was less than with DIM and BII (Fig. 2A). Both the trimers markedly decreased the 2α- and 16α-hydroxylation of testosterone to a mere 5% of the control cultures (Fig. 2B, Table 1). No competitive inhibition of the 2α- and 16α-hydroxylation of testosterone could be detected when microsomes of untreated rats were

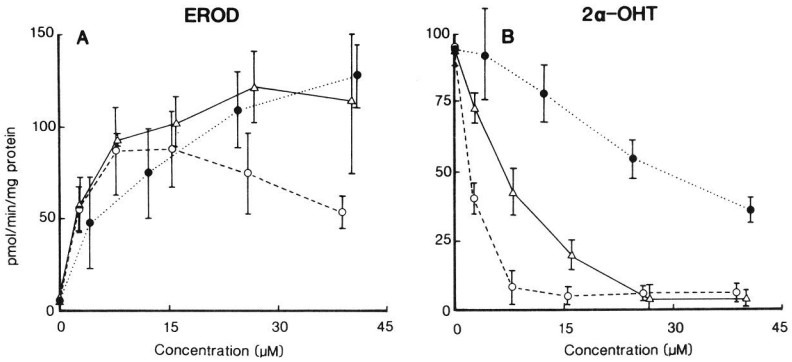

Fig. 2: EROD activity and testosterone 2α-hydroxylation (2α-OHT) in intact monolayers of primary rat hepatocytes treated with DIM (●), CTI (○), and BII (△) for 48 hr. Data are means ± SD (n = 3 experiments).

Table 1. Testosterone hydroxylation in intact monolayers of rat hepatocytes after exposure to different acid-reaction products of indole-3-carbinol.

Treatment	µg/ml	µM	15β-OHT	7α-OHT	16β-OHT	16α-OHT	6β-OHT
			(pmol/min/mg cellular protein)				
Control			7.0 ± 0.5	6.1 ± 2.1	nd	167.9 ± 5.9	33.3 ± 1.9
DIM	1	(4.1)	7.6 ± 1.6	11.5 ± 5.4	nd	164.9 ± 5.9	36.5 ± 7.6
	3	(12.2)	8.5 ± 2.1	15.1 ± 6.7*	nd	137.7 ± 9.9*	38.6 ± 7.9
	6	(24.4)	12.1 ± 0.5*	22.4 ± 9.9*	nd	105.2 ± 5.1*	44.0 ± 5.3*
	10	(40.7)	11.9 ± 2.7*	28.2 ± 12.4*	nd	77.1 ± 11.0*	53.3 ± 8.8*
CTI	1	(2.6)	10.6 ± 3.3	15.4 ± 7.4	nd	67.9 ± 24.9*	37.4 ± 13.7
	3	(7.8)	7.3 ± 1.2	14.7 ± 5.9*	nd	17.5 ± 4.8*	33.7 ± 11.5
	6	(15.5)	7.0 ± 2.5	17.3 ± 8.8*	nd	8.6 ± 3.5*	27.9 ± 12.9
	10	(25.8)	4.3 ± 0.3*	15.5 ± 3.3*	nd	6.2 ± 2.1*	18.0 ± 3.6*
	15	(38.7)	5.3 ± 2.6*	20.1 ± 4.0*	nd	4.5 ± 1.6*	22.9 ± 4.8*
BII	1	(2.7)	9.7 ± 1.4*	12.4 ± 6.5	nd	139.0 ± 19.0*	34.9 ± 6.7
	3	(8.0)	8.8 ± 2.1*	17.2 ± 8.1*	nd	87.9 ± 25.2*	34.4 ± 12.2
	6	(16.0)	9.2 ± 1.5*	21.7 ± 7.5*	nd	40.1 ± 11.1*	37.7 ± 7.9
	10	(26.7)	9.9 ± 2.8	25.4 ± 13.2	nd	17.1 ± 3.5*	34.6 ± 6.5
	15	(40.0)	6.7 ± 1.9	20.4 ± 7.6*	nd	6.4 ± 2.5*	22.6 ± 8.0*

Values represents means ± SD of three experiments. * $P \leq 0.05$ when compared to control. After a preincubation period of 24 hr, hepatocytes were exposed for another 48 hr to DMSO alone (control), 3,3'-diindolylmethane (DIM), 5,6,11,12,17,18-hexahydro-cyclonona [1,2-*b*:4,5-*b*':7,8-*b*":] tri-indole (CTI) and 2,3-bis [3-indolylmethyl] indole (BII). nd = not detectable.

incubated with both testosterone and BII or CTI (results not shown). Treatment of cells with DIM diminished this hydroxylation of testosterone, but only to *ca* 40% of the control cells. All three acid-condensation products enhanced the 7α-hydroxylation of testosterone (to *ca* 4-fold; Table 1), whereas the hydroxylation at the 15β- and 6β-site were slightly enhanced in cells exposed to DIM and BII (1.5-fold), but decreased in cells exposed to CTI (0.6-fold). Western blotting revealed enhanced P450 1A1, but not of 1A2, 2B1/2 and 3A apoprotein levels in cells treated with DIM, CTI or BII (results not shown).

Effects of indole derivatives on P450 enzymes in monkey hepatocytes

The basal P450-dependent activities in 72 hr-old monkey hepatocytes differed from the activities measured in rat hepatocytes. The basal EROD activity was about 4-fold higher than in rat hepatocytes. Also the hydroxylation at the 6β-site of testosterone was markedly higher (30-fold) in DMSO-treated monkey hepatocytes. In contrast, the

hydroxylation at the 2α-site was very low, and the formation of 7α-OHT could not be detected in these 72 hr-old monkey hepatocytes.

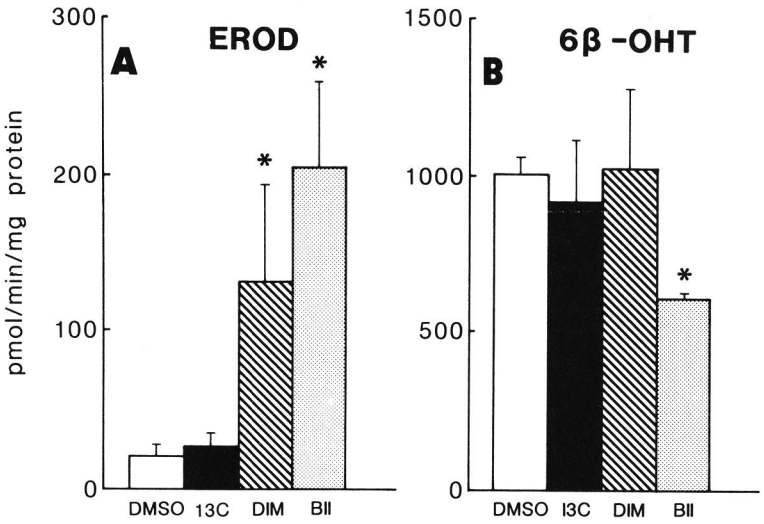

Fig. 3: EROD activity and testosterone 6β-hydroxylation (6ß-OHT) in intact monolayers of primary monkey hepatocytes treated with I3C, DIM, and BII for 48 hr. Data are means ± SD. * $P \leq 0.05$ compared to cells treated with DMSO alone (n = 3 experiments).

Table 2. Testosterone hydroxylation in intact monolayers of monkey hepatocytes after exposure to indole-3-carbinol and two acid-condensation products of indole-3-carbinol.

Treatment	(μM)	15β-OHT	7α-OHT	16α-OHT	16β-OHT	2α-OHT
		(pmol/min/mg cellular protein)				
Control	-	40.0 ± 4.4	nd	14.6 ± 5.3	39.1 ± 5.9	6.7 ± 0.6
I3C	(25)	35.0 ± 10.8	nd	12.0 ± 1.7	33.9 ± 5.5	6.2 ± 1.8
DIM	(25)	40.8 ± 12.3	nd	12.6 ± 4.7	37.9 ± 10.9	7.2 ± 2.0
BII	(25)	21.8 ± 2.0*	nd	11.5 ± 5.0	23.8 ± 4.6*	5.1 ± 1.0*

Values represents means ± SD of three experiments. * $P \leq 0.05$ when compared to control. After a preincubation period of 24 hr, hepatocytes were exposed for another 48 hr to DMSO alone (control), 25 μM indole-3-carbinol (I3C), 3,3'-diindolylmethane (DIM) and 2,3-bis [3-indolylmethyl] indole (BII). nd = not detectable.

Exposure of monkey hepatocytes to I3C for 48 hr had no effect on EROD and testosterone hydroxylation (Fig. 3; Table 2). The dimer DIM enhanced the EROD activity to 6-fold the activity in cells treated with DMSO alone, but no changes were observed in the hydroxylation of testosterone. In contrast, exposure to BII enhanced the EROD activity to 9-fold in monkey cells (Fig. 3A), whereas the formation of several metabolites of testosterone (15β-, 6β-, 16β-, 2α-OHT and androstenedione) was reduced (Fig. 3B; Table 2).

Phase II enzymes in rat and monkey hepatocytes

In rat liver cells the indole derivatives enhanced the DTD activity dose-relatedly to 3.5-, 2.6- and 5.7-fold in DIM, CTI and BII treated cells, respectively (Fig. 4A). The glucuronidation of naphthol in intact rat cells was enhanced by treatment with all three indoles, although a higher concentration of CTI and BII diminished these effects (Fig. 5A). No effects of the acid-condensation products of indole-3-carbinol were observed in rat cells on the GST activity (Fig. 4B) and sulphation of 1-naphthol (Fig. 5B).

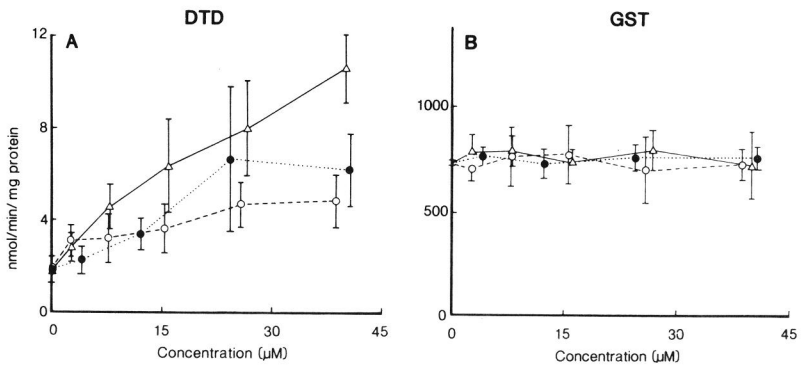

Fig. 4: DT-diaphorase (DTD) and glutathione S-transferase (GST) activity in cytosol of rat hepatocytes treated with DIM (●), CTI (○), and BII (△) for 48 hr. Data are means ± SD, n = 3 experiments.

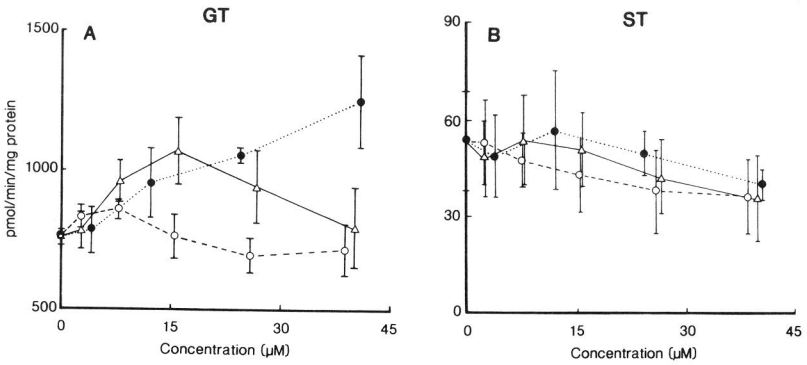

Fig. 5: Glucuronidation (GT) and sulphation (ST) of 1-naphthol in intact monolayers of primary rat hepatocytes treated with DIM (●), CTI (○), and BII (△) for 48 hr. Data are means ± SD, n = 3 experiments.

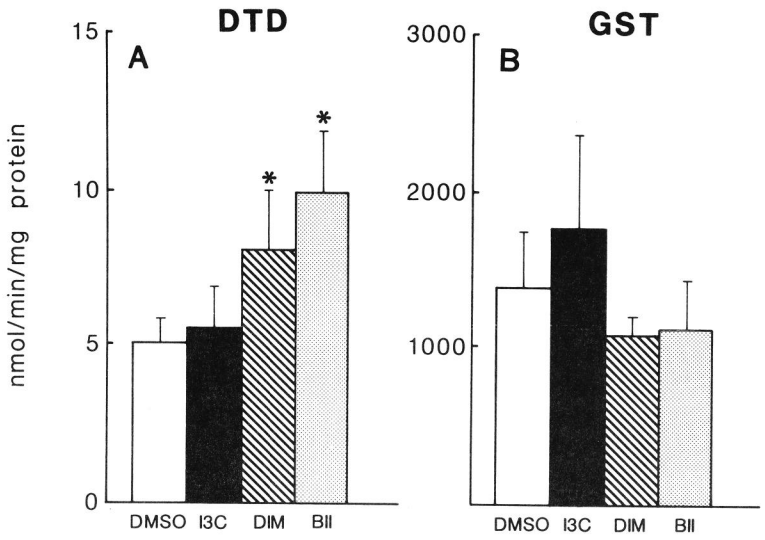

Fig. 6: DTD activity (DTD) and glutathione S-transferase activity (GST) in cytosol of cultured monkey hepatocytes, treated with I3C, DIM, and BII, respectively (48 hr). * $P \leq 0.05$ compared to cells treated with DMSO alone (n = 3 experiments).

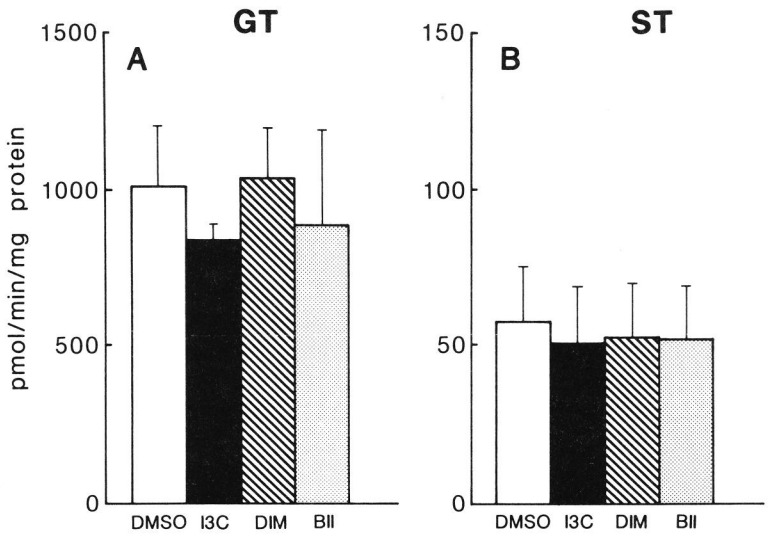

Fig. 7: Glucuronidation (GT) and sulphation (ST) of 1-naphthol in intact monolayers of primary monkey hepatocytes, treated with I3C, DIM, and BII, respectively (48 hr). * $P \leq 0.05$ compared to cells treated with DMSO alone (n = 3 experiments).

In monkey hepatocytes treated with DMSO alone the glucuronidation and sulphation of 1-naphthol, and the DTD activity did not differ from similarly treated rat hepatocytes. In contrast, the basal GST activity was twofold higher in monkey hepatocytes compared with control rat hepatocytes. Treatment of monkey hepatocytes with 25 μM BII resulted in enhanced DTD activity (2-fold; Fig. 6A). No changes were observed in GST, and glucuronidation and sulphation of 1-naphthol after treatment of monkey cells with either I3C, DIM or BII (Fig. 6B; Fig 7).

Discussion

Treatment of rat hepatocytes with the acid-condensation products of indole-3-carbinol resulted in a P450 pattern comparable to the pattern observed after treatment with the known inducer β-naphthoflavone [17]. Apart from a high induction of EROD, the indole derivatives DIM, CTI and BII markedly decreased the formation of 2α-OHT and 16α-OHT in rat hepatocytes. In the rat at least five P450 enzymes hydroxylate testosterone at the 16α site, namely 2B1, 2C11, 2B2, 2C7 and 2C13, the latter three at a rate one-tenth of that observed with 2B1 and 2C11 [25,26]. In untreated rats the levels of

P450 2B1 apoproteins are very low and the testosterone 16α-hydroxylation is predominantly attributable to 2C11 [26]. The comparable effects of the indoles on the 2α-hydroxylation of testosterone, the latter specifically catalysed by P450 2C11 [25,26], indicates that hydroxylation at both the 2α- and 16α- site of testosterone can be attributed to the constitutive 2C11. A reduction of P450 2C11 is also described after exposure to other inducers of the P450 1A subfamily ranging from 80 to 40% [15]. The trimers CTI and BII, however, decreased the formation of 2α- and 16α-OHT to barely detectable levels. A marked down-regulation of the constitutive P450s *in vivo* was also reported for the antioxidants butylhydroxytoluene and butylhydroxyanisole, although these compounds do not induce P450 1A1 [27].

All three indole derivatives increased the 7α-hydroxylation of testosterone in rat hepatocytes, which is predominantly catalysed by P450 2A [28]. A similar enhancement of this activity is observed when rat hepatocytes are treated with β-naphthoflavone [17]. The unaffected 16β-hydroxylation of testosterone indicates no effect of the indole derivatives on P450 2B [26]. In rat liver 6β- and 15β-hydroxylation of testosterone are both specifically catalysed by P450 3A [26]. However, in the present study no differences could be detected in P450 3A apoprotein levels and it is therefore not clear which P450(s) is (are) associated with the minor changes in testosterone 6β- and 15β-hydroxylation due to treatment with the indole-derivatives.

Less data are available on the different P450 forms in monkey liver. So far, a few studies have shown the high similarity between monkey and human P450s [29,30]. P450 enzymes in human liver belong to the subfamilies P450 1A, 2C, 2D, 2E, 3A and 4A [31]. Not P450 2C, but 3A is a major constitutive enzyme in human liver, which has a high catalytic activity towards the 6β site of testosterone [32], which was also observed in the present study. The hydroxylation of other sites of testosterone was minimal and probably not specifically related to different P450 forms [32]. On the other hand, the dealkylation of ethoxyresorufin is catalysed by the P450 1A subfamily in both rat and human hepatocytes, although in human liver mainly the P450 1A2 form is detectable [33]. In agreement with rat hepatocytes [8], I3C itself had no effects on any of the measured biotransformation activities in monkey hepatocytes. The enhancement of EROD due to exposure to BII, and an overall decrease in testosterone hydroxylation are in agreement with the effects observed in monkey hepatocytes treated with the P450 1A1 modelinducer, β-naphthoflavone (Mennes, unpublished results).

Treatment of hepatocytes with the acid condensation products of I3C selectively affected phase II enzymes. In a previous study [13], we reported enhancement of DTD activity in rat cells after exposure to 25 μM of either DIM, CTI or BII. Here, we report a clearly dose-related enhancement of DTD and GT activities in rat hepatocytes treated with BII and DIM. The selective inhibition of BII at concentrations above 15 μM, may indicate a competition between the substrate 1-naphthol and BII (or its metabolites) for the GT enzyme. A similar selective inhibition was measured for DIM and EROD activity at a concentration above 15 μM. The mechanisms behind these decrease in enzymatic activities are not clear.

Several studies suggest a concomitantly induction of P450 1A1 and phase II enzymes, such as DTD and GT by planar aromatic structures via binding of the inducer

to a cytosolic receptor, the *Ah* receptor [34,35]. In this respect, the mechanism by which the indole derivatives elicit their broad effects on biotransformation enzymes might be under control of this *Ah* receptor. Gillner *et al* [36], however, reported a relatively low binding affinity of DIM to the *Ah*-receptor, in contrast to the high binding affinity of ß-naphthoflavone, and of a more planar indole, indolo[3,2-*b*]carbazole. Metabolism of DIM to a compound more capable in binding to the *Ah* receptor has been suggested [36]. Despite its planar structure, CTI does not fit into the assumed rectangle which has been suggested to account for the binding of high-affinity ligands to the *Ah* receptor [37]. However, no *Ah* binding affinities of the trimers itself and/or possible metabolites are determined in this study. In addition, the differential induction of GT and DTD indicates that other mechanisms than the *Ah* receptor are probably involve, too [34].

Exposure of rats to I3C *in vivo* is correlated with marked changes in biotransformation activities in liver and small intestinal mucosa [4,8,9,10]. As I3C exposure is associated with a decreased tumour formation induced by a variety of chemically distinct compounds [2,4], it has been postulated that an increased first-pass metabolism in the gastro-intestinal tract could result in a reduced absorption of the precarcinogen [8,38]. In addition, enhanced detoxication of precarcinogens by either phase I or phase II enzymes in the liver and/or other target organs has also been suggested [1,2]. In this respect, the effects of indole derivatives on changes in P450 profile as well as on DTD strengthen the latter hypothesis. Many anticarcinogens such as phenolic antioxidants and 1,2-dithiol-3-thiones induce the DTD activity [39]. Moreover, the last few years the contribution of many individual P450s, including constitutive P450s, in the formation of the proximate carcinogenic is studied in more detail. For example, aflatoxin B_1 and dimethylbenz[*a*]anthracene, both compounds often used to show the anticarcinogenic effects of cruciferous vegetables and I3C *in vivo* [2,4,38], are metabolically activated by the constitutive P450 forms of the subfamily P450 2C, whereas P450 1A1 enhances the formation of the non-mutagenic metabolite [40,41]. However, the effects of indole-derivatives on both cytochrome P450 profile and several phase II enzymes, makes it possible that conditions may exist in which enhancement of carcinogenesis may occur. Furthermore, the formation of tumours is a multi stage process and apart from metabolic activation other factors such as species-difference and tissue-specific expression biotransformation enzymes in target organs can all affect the ultimate risk of tumour formation.

In general, our study identifies the acid condensation products of I3C, DIM, CTI and BII, as potent modulators of several biotransformation activities. The fact that similar effects are observed in rat as well as in monkey hepatocytes indicate that these indole oligomers could be of importance for humans, too. However, the relevance of these changes in biotransformation on the modulation of carcinogenesis by indoles remains to be established.

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CHAPTER 8

GENERAL DISCUSSION

Biotransformation of xenobiotics may influence the toxicity of several xenobiotics. Changes in activities of biotransformation enzymes may therefore drastically affect the subceptibility of organisms to these chemicals. Compounds with widely distinct chemical structures have been shown to be either potent inducers or inhibitors of biotransformation enzymes. Through the intake of diet, drugs and air, organism are exposed to many different compounds. Knowledge of the effects of all these various chemicals on biotransformation enzymes is only limited. Generally, the induction potential of chemicals is determined via exposure of living animals (mainly rodents) to a chemical. Per one single compound and concentration a minimum of three animals (mainly rodents) are treated with the test chemical for several days. Subsequently biotransformation enzyme activities are measured in cellular fractions derived from the liver and, if required, other organs. To reduce the number of animals in such tests, and to decrease the level of distress to the animals, *in vitro* models with primary cultures of hepatocytes may offer a good alternative for at least part of these animal studies.

The main objective in this thesis is the application of primary hepatocyte cultures to determine the potential of compounds to change cytochrome P450 enzymes. Simple and convenient methods were developed to determine P450-dependent catalytic activities directly in intact monolayers (chapter 2). Thereafter inducers known to be effective *in vivo* were used to "validate" the *in vitro* model system (chapter 3). In the last four chapters the *in vitro* model was applied, complementarily to *in vivo* studies, to investigate the effects of a major breakdown product of glucosinolates, i.e. indole-3-carbinol (I3C) on the activities of biotransformation enzymes. In this chapter the most important results will be highlighted and generally discussed.

I. Changes in cytochrome P450 enzyme pattern in primary cultures of rat hepatocytes

As indicated in the Introduction and specified in chapter 2, the total cytochrome P450 content but also the individual forms rapidly decrease during primary culture of rat hepatocytes. In contrast to previous suggestions that qualitative changes in the P450 profile may occur during culture, in favour of P450 1A1, no gross changes in the P450-dependent catalytic profile was observed in our study. A possible explanation is the use of more specific substrates to define the P450 profile in more detail.

One of the main advantages of using hepatocytes over the living animal is the fact that cells isolated from one liver can be divided over many experimental units, and as such can be used to test several chemicals and/or concentrations. Generally cellular fractions (microsomes and cytosol) are prepared from cultured cells to study specific catalytic enzyme activities. However, due to a very low recovery (30%) of cytochrome P450, and an overall decline of cytochrome P450 during culture, relatively high amounts

of cells were needed to determine P450-dependent catalytic activities. Simple and convenient methods were developed to determine the P450-dependent catalytic activities directly in intact monolayers of hepatocytes. A major advantage of using intact cells over cellular fractions is the study of enzymatic reactions under physiological microconditions. Therefore, we consider these enzyme assays in intact cells more representative to the "*in vivo*" situation, although the uptake and transport, and possible conjugation reactions should be taken into consideration.

In chapter 3 several inducers known to be effective *in vivo* were chosen to study changes in the P450 pattern in primary hepatocyte cultures. It appeared that in such cultures several P450 enzyme activities (i.e. 1A1/1A2, 2A, 3A and 4A) can be elevated with the same inducing compound and to the same extent as *in vivo*, while decrease of P450 2C could also be detected. The induction of P450 2B1/2 by phenobarbital (PB), however, as measured at the level of mRNA, protein and activity (PROD and 16β-hydroxylation of testosterone) occurs at a much lower level than *in vivo*. A similar low induction of this P450 form and associated activities were observed when cells were treated with 22'44'55'hexachlorobiphenyl, which is another potent inducer of P450 2B1/2 *in vivo* (Jansen, unpublished results). In contrast, clofibrate induced the P450 2B1/2 catalytic activity (PROD and 16β-OHT) to a similar level *in vivo* as *in vitro*. PB (known to induce GST *in vivo*) enhanced the GST activity *in vitro*, too (Jansen, unpublished results), indicating a selective effect of PB on P450 2B1/2. Several attempts were made to affect the induction of P450 2B, using either serum-free media, low oxygen pressure, additional haem, or an additional minor concentration of P450 1A1-inducer, but none were successful (Wortelboer, unpublished results).

The results indicate that primary cultures of rat hepatocytes cultured on plastic retain the capacity to respond to PB induction, however, in the case of P450 2B1/2 to a much lesser extent than *in vivo*. The fact that clofibrate does induce P450 2B1/2 *in vitro* to a similar level as *in vivo* suggests a selective mechanism of regulation for this P450 enzyme by the moderate inducer clofibrate compared to the more potent inducers PB and 22'44'55'hexachlorobiphenyl.

Another P450 enzyme which could not be induced in primary rat hepatocytes cultured on plastic, is P450 2E. The induction of this isoenzyme *in vivo* is correlated with ligand binding and stabilization of mRNA levels, which subsequently results in higher protein levels (Song *et al*, 1989). The reason for the lack of induction of this P450 form *in vitro* is not clear.

Recently, promising new findings have been reported for the induction of P450 2B1/2 and 2E in hepatocytes when cultured on different matrices. Treatment of hepatocytes cultured on an extracellular tumour matrix (Matrigel) in serum-free Williams E medium with PB or ethanol enhanced apoprotein levels as well as the catalytic activities of P450 2B or P450 2E, respectively (Sinclair *et al*, 1990; Sinclair *et al*, 1991). No direct comparisons were made with induction *in vivo*, but the enhancement of the P450 2B-associated catalytic activity (pentoxyresorufin dealkylation) in cells treated with PB was very high (*ca* 30-fold; Sinclair *et al*, 1990) in comparison with our system. The P450 2E apoprotein levels as well as its associated catalytic activity could be induced when cells cultured on matrigel were treated with ethanol. Matrigel is a mixture of

components extracted from the Engelbreth-Holm-Swarm tumour. The tumour has to be passaged and maintained in *ca* 10-week-old mice (Schuetz *et al*, 1988). Therefore, such a procedure does not fit into an *in vitro* approach directed towards a reduction in animal distress. Furthermore, the extraction of the tumour is very laborious and time consuming, and therefore no satisfying solution for a "rapid" (cheap) screening test for induction.

Another method is to pre-coat dishes with a matrix which is commercially available, i.e. Vitrogen (type 1 collagen). Sinclair *et al* (1990) reported an enhancement of pentoxy-resorufin dealkylation (*ca* 25-fold) in hepatocytes treated with PB from 48 to 96 hr after isolation. To date no data are available whether P450 2E is inducible in hepatocytes cultured on Vitrogen. Waxman *et al* (1990) prolonged the viability of hepatocytes for a full 5 weeks, when cells were cultured on Vitrogen-coated plates into a serum-free modified Chee's medium (for composition see Waxman *et al*, 1990). Exposure of cells to PB enhanced P450 2B apoprotein levels and associated activities up to levels nearly as high as those achieved in PB-induced rat liver *in vivo*.

A totally different approach to extending functionality of cultured hepatocytes is the use of co-cultures of primary hepatocytes with rat liver epithelial cells (Guguen-Guillouzo *et al*, 1983). Although the life time of the primary cultures was extended and total cytochrome P450 could be maintained at higher levels than in conventional cultured cells, the enhancement of P450 2B-associated activities in co-cultured cells treated with PB compared to untreated cells was still very low (Rogiers *et al*, 1990). The supporting cell culture in this system is not an established cell line and the cells have to be isolated once in a while. For this reason, the use of epithelial-like cell lines would be favoured. The effects of several cell-lines in co-culture with hepatocytes are currently under investigation (Kuri-Harcuch and Mendoza-Figueroa, 1989; Donato *et al*, 1990a; Donato *et al*; 1990b). However, up to now, the induction of P450 2B in these co-cultures is limited too (Donato *et al*, 1990b).

From the foregoing it is obvious that many laboratories are involved in studying the problem of the low induction of P450 2B and P450 2E *in vitro*. Further research on the regulation of the expression of these genes, and additional efforts to search for better culture conditions would add further useful information.

We conclude that up to now primary cultures of hepatocytes cultured on plastic can not be used as a complete "replacement test", in replacing *in vivo* induction studies (Balls *et al* 1990). Despite this incompleteness of the *in vitro* model today, hepatocyte cultures can be used as a "screening test" for the induction and suppression of P450 1A1, 1A2, 2A, 2C11, 3A and 4A: i.e. a simple and rapid test useful for making preliminary decisions among large groups of chemicals in the selection for further testing, either with intact animals or more-sophisticated *in vitro* tests. For example, to study the induction potential and/or induction potency of chemicals for P450 1A1, as in the case of e.g. PCDDs and PCDFs, primary cultures of rat hepatocytes are currently used to study dose-response relationships of these compounds. To reduce the amount and distress of animals in laboratory experiments ("reduction" and "refinement"), primary cultures of liver cells may be used wherever possible in conjunction with a limited number of *in vivo* studies.

II. Application of the in vitro model complementarily to in vivo studies: The effects of indole-3-carbinol (I3C) on biotransformation enzymes

In the second part of this thesis the *in vitro* model is applied in the study of the effects of some important food constituents. Cruciferous vegetables have been shown to enhance the activity of biotransformation enzymes in both animals and man. These vegetables contain appreciable amounts of glucosinolates which upon hydrolysis yield various substances. The breakdown products of the indole glucosinolates, e.g. indole-3-carbinol are associated with marked changes in biotransformation enzyme activities, but in addition can markedly affect tumour formation.

In chapter 4 and 5 we studied the time- and dose-related effects of cooked Brussels sprouts and one of the major hydrolysis products of indole-glucosinolates, i.e. I3C, on the changes in biotransformation enzymes in rat liver and small intestine *in vivo*. Two days of exposure to 5% Brussels sprouts as well as 200 mg/kg (0.02%) I3C already changed several biotransformation enzymes. The common effect of both Brussels sprouts and I3C is the enhancement of P450 2B apoprotein levels and associated activities in the small intestine. Recently, Vang *et al* (1991) reported a comparable enhancement of P450 2B apoprotein levels in the colon tissue of rats exposed to a diet containing 10% broccoli. On the other hand, markedly different effects between the vegetable as a whole and I3C were also observed. In the liver Brussels sprouts predominantly affected P450 1A2, whereas I3C affected both P450 1A1 and 1A2; Brussels sprouts (from 5% upwards) enhanced both GST, GT1, and DTD (the latter over 3-fold in the small intestine), whereas only the high dose of I3C (500 mg/kg diet) affected phase II enzymes. These results stress the effects of other constituents of Brussels sprouts. For instance, goitrins and dithiolthiones are both known to affect predominantly the phase II enzymes, especially GST and DTD (Chang and Bjeldanes, 1985; Ansher *et al*, 1986).

In contrast to the effects of I3C *in vivo*, treatment of hepatocytes with I3C resulted only in minor changes in biotransformation activities. On the other hand, 3,3'-diindolylmethane (chapter 5) and acid-treated I3C (Wortelboer *et al*, 1991) markedly induced P450 1A1 and EROD activity *in vitro*. Therefore, we studied the condensation of I3C under acidic conditions (chapter 6). I3C is rapidly converted to several oligomers of methylene-indole. Next to the dimer 3,3'-diindolylmethane (DIM) two other major products (trimers) were formed. The chemical structures could be elucidated using nuclear magnetic resonance: the cyclic trimer 5,6,11,12,17,18-hexahydro-cyclonona [1,2-*b*:4,5*b'*:7,8-*b''*] tri-indole (CTI) and the non-cyclic trimer 2,3-bis [3-indolyl-methyl] indole (BII). The condensation of I3C is pH-dependent and CTI could not be detected at a pH > 4.5. Treatment of rats with I3C by gavage confirmed the formation of DIM and BII in the stomach, though CTI could not be detected. The latter is probably due to the relatively high pH of the rat stomach (pH 4-6), in comparison with the pH of the human stomach. Therefore, it seems likely that at least all three indole oligomers are to be formed out of I3C in the human stomach.

To determine whether DIM, CTI and BII have the potential to change biotransformation enzyme activities, we studied these effects in primary cultures of rat hepatocytes (chapter 7). All three indole derivatives induced P450 1A1 and DTD activities, BII being the most potent one. On the other hand, the formation of 2α- and 16α-hydroxytestosterone was markedly suppressed (to a mere 5% of the activities in

untreated cells). Since we did not have the disposal of highly specific antibodies directed towards P450 2C11, no apoprotein levels of this P450 form could be measured. However, we suggest that the decrease in the formation of 2α and 16α hydroxylation is attributable to a down regulation of the male constitutive P450 2C11. *In vivo*, however, dietary exposure to I3C did not decrease the hepatic hydroxylation of testosterone at either the 2α or the 16α site (chapter 5). Differences in concentration of the effective compound *in vivo* and *in vitro* may explain these differences. Furthermore, I3C exposure *in vivo* also induced the hepatic P450 2B1 apoprotein levels (chapter 5). P450 2B1 also hydroxylates testosterone at the 16α site (Wood *et al*, 1983) and therefore could mask a possible decrease in P450 2C11.

An important feature of *in vitro* models is that cultured cells can be used to improve interspecies extrapolation, e.g. in comparing the effects of compounds in rat liver cells with human liver cells. However, due to the increasing number of liver transplantations, human liver is only rarely available for cell isolation. As an alternative to human liver cells, we used hepatocytes derived from cynomolgus monkey liver (*Macaca fascicularis*) to study the effects of I3C, DIM and BII on biotransformation enzymes (chapter 7). I3C did not affect any biotransformation activity measured in monkey hepatocytes. Although the overall effect was lower in monkey cells compared to the effects in rat cells, BII enhanced both EROD as well as DTD activities. Furthermore, BII decreased the 6ß-hydroxylation of testosterone, which is predominantly catalysed by the human constitutive P450 form, P450 3A. These results, in addition to the probable formation of these indole-derivatives due to low pH in the human stomach, indicate that the acid condensation of I3C could be of importance for humans, too.

The question arises whether the levels used in the *in vivo* studies are comparable to the human intake of indoles via the consumption of cruciferous vegetables. In this respect, the mean daily intake of indolylmethyl glucosinolate has been estimated to be 7 mg/person from cooked cruciferous vegetables (Sones *et al*, 1984). Assuming a daily consumption of 500 g total dry substance, a daily ingestion of 7 mg glucobrassicin would represent 1.4% of the dry matter of the total diet. In the studies presented in this thesis we used 0.02% and 0.05% I3C. In addition, a daily consumption of 150 g cooked Brussels sprouts (with a dry matter content of 15%) would represent 4.5% of the dry matter of the total diet. The results presented in this thesis indicate that a diet of 2.5% cooked Brussels sprouts already induced P450 2B apoprotein in the small intestine. Clearly, the levels used in this study could be those normally encountered in the human diet.

An increased consumption of either cruciferous vegetables or I3C by rats is generally associated with a decreased risk of tumorigenesis induced by a wide variety of precarcinogens, such as aflatoxin B_1, benzo[*a*]pyrene, 7,12-dimethylbenz[*a*]anthracene (McDanell *et al*, 1988; Salbe and Bjeldanes, 1989). On the other hand, enhanced tumorigenesis in cabbage-fed animals has also been reported for 1,2-dimethylhydrazine (Srisangnam *et al*, 1980) and *N*-nitrosobis-(2-oxopropyl)amine (Birt *et al*, 1987). The results in chapter 4 and 5 indicate that Brussels sprouts and I3C do not have a common change in biotransformation activities which may affect the carcinogenic process. In this respect, exposure to Brussels sprouts have a profound effect on P450 2B, GST and DTD

activities in the small intestine, which may result in a higher first pass elimination of chemicals in the small intestine, thereby reducing the absorption of the chemical in the blood. Interestingly, in both studies in which enhancement of tumorigenesis has been reported the pre-carcinogens were administrated subcutaneously (instead of orally), thereby bypassing the gastrointestinal tract. The enhancement of hepatic GST and DTD are both in favour of detoxifying route of pre-carcinogens.

In the case of I3C, however, the effects on intestinal P450 activities are relatively low in comparison to the activities measured after dietary exposure of Brussels sprouts, and more selective mechanisms can not be ruled out. For example, the enhancement of DTD activity is observed with several other compounds known to decrease the risk of chemically-induced tumorigenesis, such as BHA and BHT (Benson *et al*, 1980), and dithiolthiones (De Long *et al* 1986). In addition, changes in the P450 profile in favour of the detoxication of the precarcinogen could affect the carcinogenic potential, too. In this regard, a high induction of P450 1A1 in the liver as observed after I3C treatment is often correlated with a potential higher risk of bioactivation of precarcinogens (Ioannides and Parke, 1990). However, in many other studies exposure to P450 1A1 inducers *in vivo* decreased chemically- induced tumorigenesis, in contrast to a higher bioactivation and associated higher DNA-adduct formation or mutagenicity using cellular fractions, e.g. microsomes (Williams *et al*,1989; McKillop *et al*, 1991). Nevertheless, the fact that I3C affects both cytochrome P450 and several phase II enzymes, may explain that conditions can exist in which enhancement of carcinogenesis may occur. Furthermore, carcinogenesis is a multi-stage process and many additional factors different from bioactivation may affect the formation of tumours.

In conclusion, the results described in this thesis clearly illustrate the wide applicability of primary cultures of hepatocytes in induction studies. Apart from the use of this *in vitro* model in "screening tests", primary hepatocyte cultures can be used as "adjunct tests": i.e. tests in conjunction with *in vivo* tests, to elucidate possible mechanisms of toxicity or to further investigate specific observations. However, further research is needed to improve the applicability of cultured hepatocytes as an *in vitro* model with regard to the induction and regulation of all P450 forms.

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Samenvatting voor "niet-vakgenoten"

Mens en dier krijgen dagelijks vele natuurlijke en door de mens gemaakte stoffen binnen via voedsel, inademen van vervuilde lucht, geneesmiddelen enz. Het merendeel van deze lichaamsvreemde stoffen (xenobiotica) wordt in ons lichaam via de stofwisseling dusdanig veranderd (biotransformatie), zodat deze stoffen gemakkelijk worden uitgescheiden via urine, faeces en uitgeademde lucht. Het belangrijkste orgaan waarin deze omzetting van stoffen plaatsvindt is de lever, maar ook in de darm, nieren, longen en huid kunnen lichaamsvreemde stoffen omgezet worden. Bij de biotransformatie zijn verschillende enzymen betrokken.

In hoofdstuk 1 van dit proefschrift wordt een overzicht gegeven van de belangrijkste enzymen (cytochroom P450, conjugatie enzymen, DT-diaphorase) die bij de biotransformatie van lichaamsvreemde stoffen betrokken zijn. De omzetting van stoffen gebeurt vaak in twee stappen: de lichaamsvreemde stof wordt eerst veranderd door cytochroom P450 enzymen (fase I) om dan vervolgens door conjugatie enzymen veranderd te worden (fase II) zodat het gevormde produkt het lichaam gemakkelijk kan verlaten.

Er zijn echter ook stoffen bekend die in ons lichaam door dezelfde enzymen omgezet kunnen worden tot schadelijke produkten (ook wel bioactivatie genoemd). De gevormde giftige produkten (toxische metabolieten) kunnen beschadiging aan weefsel en DNA geven, waardoor o.a. tumoren kunnen ontstaan. In het algemeen worden deze toxische metabolieten weer verder gebiotransformeerd door enzymen tot inactieve (= niet toxische) produkten, zodat geen schade aan weefsels kan ontstaan. Het zal duidelijk zijn dat een toxisch effect dan ook afhangt van de balans tussen de bioactivatie en inactivatie.

Er zijn echter ook stoffen bekend die de hoeveelheid en snelheid van deze biotransformatie enzymen (cytochroom P450, conjugatie enzymen) kunnen veranderen. Onderzoek heeft aangetoond dat niet alleen allerlei geneesmiddelen en giftige stoffen zoals PCBs, dioxines etc. de aktiviteit van deze enzymen kunnen veranderen, maar ook allerlei stoffen die in onze voeding voorkomen. De consequentie van deze veranderingen kan zowel gunstig als ongunstig zijn. Als bijvoorbeeld de hoeveelheid en snelheid van "activerende" enzymen verhoogd wordt, dan zal het duidelijk zijn dat de kans op vorming van giftige produkten groter zal zijn en er een grotere kans op het ontstaan van kanker zal zijn. Echter, als bijvoorbeeld de hoeveelheid en snelheid van "inactiverende" enzymen verhoogd wordt dan kan dit een beschermende werking hebben tegen kankerverwekkende stoffen, omdat de giftige produkten sneller weggevangen worden. Het is juist de balans tussen deze activering en inactivering die de toxiciteit van vele verbindingen bepaalt.

We weten eigenlijk van ontzettend veel stoffen nog niet of ze de snelheid van deze enzymen kunnen veranderen. Tevens weten we ook nog niet waarom de ene mens wel en de andere mens geen kanker krijgt. Wel is langzamerhand duidelijk dat factoren zoals dieet, roken, bepaalde geneesmiddelen etc. het risico op het krijgen van kanker kunnen beïnvloeden. De beïnvloeding van dit soort stoffen op de balans van activerende en inactiverende enzymen kan hierin een grote rol spelen.

Dit proefschrift

Om te onderzoeken of een stof in staat is om fase I en/of fase II enzymen te veranderen worden vaak ratten gebruikt als proefdier model voor de mens. Nadat de dieren een aantal dagen een bepaalde stof hebben gekregen, wordt vervolgens de lever (en eventueel andere organen) uit het dier gehaald, zodat de activiteit van de enzymen in de organen gemeten kan worden. Per dier kan je zo slechts één stof en één concentratie uittesten. Dit soort onderzoek kost veel dieren en kan bij bepaalde stoffen pijnlijk en belastend zijn voor het dier. Een alternatief hiervoor is om cellen uit de lever van één dier te isoleren (*ca* 500 miljoen cellen) en deze over plastic kweekbakjes te verdelen (4 miljoen per bakje), zodat elk bakje met een andere stof en/of concentratie getest kan worden.

In de eerste twee hoofdstukken zijn experimenten beschreven waarin vergeleken wordt of de levercellen (*in vitro*) precies hetzelfde reageren als het gehele dier (*in vivo*) op stoffen waarvan bekend is dat ze de enzymaktiviteit kunnen beinvloeden. Tevens konden zo allerlei bepalingsmethoden aangepast worden zodat ook de lage enzymaktiviteit in maar een klein aantal cellen toch goed gemeten kon worden. Het is gebleken dat het *in vitro* systeem voor een aantal belangrijke vormen van cytochroom P450 een goed alternatief kan zijn voor *in vivo* onderzoek, echter niet voor alle vormen. Tot nu toe is het dan ook alleen bruikbaar als aanvulling op *in vivo* onderzoek, om het mechanisme verder uit te zoeken van stoffen waarvan *in vivo* al enigszins duidelijk is wat ze doen.

Een voorbeeld van zo'n onderzoek is de studie naar de effecten van het eten van kool (o.a. spruitjes), en meer specifiek van één stof in deze groenten (indole-3-carbinol) op veranderingen in enzymaktiviteit in de lever en de darm (laatste vier hoofdstukken). Waar mogelijk werd het onderzoek met proefdieren (*in vivo*) aangevuld met onderzoek in levercellen (*in vitro*). Uit de experimenten kwam naar voren dat de stof indole-3-carbinol door de zure omgeving in de maag omgezet wordt tot vervolgprodukten. Het bleek dat niet de stof indole-3-carbinol de activiteit van enzymen verandert, maar de zure produkten van deze stof.

In toxicologisch onderzoek wordt veelal de rat als proefdier gebruikt. Op basis van deze onderzoeksgegevens worden conclusies en normen geformuleerd voor de mens. Steeds meer ervaring wordt verkregen in het isoleren en in cultuur brengen van cellen uit organen van verschillende diersoorten. Hierdoor wordt het mogelijk om directe vergelijkende studies te doen tussen verschillende diersoorten, inclusief de mens. Door de enorme toename van orgaantransplantaties is het moeilijk om aan menselijke levercellen te komen, maar een goed alternatief zijn de levercellen van een aap. Op het Rijks Instituut voor Volksgezondheid en Milieuhygiëne moet tweewekelijks een aap opgeofferd worden voor het bereiden van het polio-vaccin. Hiervoor worden de niercellen gebruikt; andere organen (ogen, schildklier, maag etc.) worden eveneens gebruikt voor onderzoek in verschillende laboratoria. In dit onderzoek is gebruik gemaakt van de levercellen van de aap. De sterke effecten van de zure produkten van indole-3-carbinol op de enzymaktiviteiten in de levercellen van de rat vinden we in grote lijnen, maar dan bij andere enzymen, ook terug in de levercellen van de aap.

De consequentie van de veranderingen in enzymaktiviteiten kunnen, zoals al eerder aangegeven, zowel gunstig als ongunstig zijn. Studies naar de effecten van deze veranderingen op bijvoorbeeld tumorvorming of orgaanschade zijn voor proefdieren zeer belastend en vanwege de duur van de proef voor veel laboratoria te kostbaar. Om dergelijke vragen verder deels met behulp van levercellen te kunnen onderzoeken in plaats van in proefdieren, zullen nog aanvullende (met name gevoelige) methodes ontwikkeld moeten worden.

Curriculum vitae

Heleen Wortelboer werd op 17 mei 1958 te Den Helder geboren. In 1976 behaalde zij het gymnasium-β diploma aan het R.K. Lyceum Sancta Maria te Haarlem. Twee en een half jaar is zij werkzaam geweest als radio-diagnostisch laborante in het R.K. Ziekenhuis Maria Stichting te Haarlem. In 1979 begon zij aan de studie Biologie aan de Vrije Universiteit te Amsterdam, waar de bijvakken Plantenoecologie (Prof. Dr. W.H.O. Ernst) en Dierfysiologie/Endocrinologie (Prof. Dr. J.J. Joosse) werden gevolgd. Binnen het hoofdvak Toxicologie (Prof. Dr. W. Seinen) is onderzoek gedaan bij de vakgroep Antropogenetica (Dr. H. Joenje, Amsterdam) en de sector Biologische Toxicologie (Dr. B.J. Blaauboer, Utrecht). Naast deze wetenschappelijke opleiding werd de eerstegraads onderwijsbevoegdheid behaald. In 1987 werd het doctoraalexamen Biologie afgelegd aan de Rijks Universiteit Utrecht.
Vanaf mei 1987 tot april 1991 was zij als promovendus werkzaam binnen het UTOX, een samenwerkingsverband tussen het Research Instituut voor Toxicologie (RITOX) te Utrecht, TNO-Voeding (TNO-ITV) te Zeist en het Rijks Instituut voor Volksgezondheid en Milieu (RIVM) te Bilthoven. Het promotieonderzoek werd uitgevoerd binnen het RITOX onder leiding van Dr. B.J. Blaauboer en binnen TNO-Voeding onder leiding van Dr. H.E. Falke. Naast het onderzoek is de postdoctorale opleiding Toxicologie gevolgd.

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Bedankt !

Natuurlijk is dit proefschrift niet alleen tot stand gekomen door de inzet van degene wiens naam op de omslag staat vermeld.

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